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Effects of 17α-Ethinylestradiol (EE_2) on Reproductive Endocrine Status in Mummichog (*Fundulus Heteroclitus*) Under Differing Salinity and Temperature Conditions

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EFFECTS OF 17α-ETHINYLESTRADIOL (EE_2) ON REPRODUCTIVE ENDOCRINE STATUS IN MUMMICHOG (*FUNDULUS HETEROCLITUS*) UNDER DIFFERING SALINITY AND TEMPERATURE CONDITIONS

by

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Abstract

Waterborne exposure to 17α-ethinylestradiol (EE_2), a synthetic estrogen, has previously been shown to decrease reproductive endocrine status in the estuarine killifish or mummichog (*Fundulus heteroclitus macrolepidotus*; northern subspecies). To evaluate if variations in salinity or temperature holding conditions modify the effects of EE_2 on gonad size, plasma reproductive steroid levels, and gonadal steroidogenesis, mummichog were exposed *in vivo* for 14 days to 0, 50 and 250 ng/L EE_2 in 0, 16 and 32 ppt salinity at 18°C and to 0 and 250 ng/L EE_2 at 10, 18 and 26°C at 16 ppt salinity. Effects due to salinity were limited; however, 250 ng/L EE_2 decreased plasma 17β-estradiol (E_2) levels and *in vitro* gonadal E_2 production and plasma 11-ketotestosterone (11-KT) across all salinities. Higher temperatures triggered gonadal growth in both sexes as well as increased plasma E_2 and gonadal E_2 production in females, while 11-KT production was decreased in males. EE_2 counteracted the effect of temperature as determined by depressed gonadal growth in males. In both exposures, the effects of EE_2 on testosterone (T) production were variable. The use of steroidogenic precursors (25-OH cholesterol, and/or pregnenolone and/or testosterone) in the *in vitro* gonadal incubations indicated decreased E_2 production in females and 11-KT production in males were predominately due to suppression of the terminal conversion step between T and E_2 or 11-KT. Ovarian cyp19a gene expression was not affected by 250 ng/L EE_2 compared to controls at 16 ppt and 18^{o}C (the only treatment combinations tested). The lack of effects of salinity could be protective for a species spawning in such a variable environment. Gonadal growth at higher temperatures confirms previous work on northern mummichog while EE_2 effects

on gonadal growth could be due to temperature-related increases in EE_2 uptake and/or increased susceptibility during gonadal maturation. In conclusion, the present work demonstrated that environmental conditions impact effects of EE_2, including terminal steroid production in the gonads. These results should be considered in designing standardized estuarine fish reproductive bioassays and in understanding the potential effects of reproductive contaminants in estuarine environments.

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3β-HSD	=	3β-Hydroxysteroid dehydrogenase
11-KT	=	11-Ketotestosterone
11β-HSD	=	11β-Hydroxysteroid dehydrogenase
17β-HSB	=	17β-Hydroxysteroid dehydrogenase/17β-dehydrogenase
17,20βP	=	17,20β-Dihydroxy-4-pregnen-3-one
25C	=	25-OH Cholesterol
ANOVA	=	Analysis of variance
ANCOVA	=	Analysis of covariance
Ba	=	Basal BW = Brackish water
cAMP	=	Cyclic adenosine mono-phosphate
CYP17A1	=	17,20-Desmolase
CYP19a	=	Cytochrome P450 aromatase A
CYP19b	=	Cytochrome P450 aromatase B
d	=	Days
DHEA	=	Dehydroepiandrosterone
DO	=	Dissolved oxygen
E_2	=	17β-Estradiol
EDSs	=	Endocrine disrupting substances
EE_2	=	17α-Ethinylestradiol
EF1a	=	Elongation factor-1 alpha
EIA	=	Enzymatic immunoassay
ER	=	Estrogen receptor

FSH	=	Follicle stimulating hormone
FW	=	Fresh water
GSI	=	Gonadosomatic index
GnRH	=	Gonadotropin releasing hormone
h	=	Hours
HPG	=	Hypothalamic-pituitary-gonadal
IBMX	=	3-Isobutyl-1-methylxanthine
LSI	=	Liver somatic index
LH	=	Luteinizing hormone
LOG	=	Data log-transformed prior to statistical analysis
NP	=	Post hoc test used even if normality for two-way .ANOVA failed
P	=	Pregnenolone
P450arom	=	Cytochrome P450 aromatase
P45017α	=	Cytochrome P450 17α-hydroxylase/17,20 lyase
P450scc	=	Cytochrome P450 side-chain cleavage
PDEs	=	Phosphodiesterases
PKA	=	Protein kinase A
ppt	=	Parts per thousand
RIA	=	Radioimmunoassay
RT	=	Reverse transcription
T	=	Testosterone
Sa	=	Salinity
SCP_2	=	Sterol Carrier protein 2

SEM	=	Standard error of the mean
StAR	=	Steroidogenic acute regulatory protein
STP	=	Sewage treatment plant
SW	=	Salt water

Chapter 1

General Introduction

1.1. Endocrine disrupting substances

Endocrine disrupting substances or chemicals (EDSs or EDCs) are exogenous compounds, natural or anthropogenic, which affect the physiology and morphology of fish (Länge *et al.*, 2001; Boudreau *et al.*, 2004; Peters *et al.*, 2007). Reproductive EDSs mimic, enhance or block the action of endogenous hormones causing changes to the reproductive system (Damstra *et al.*, 2002; Denslow & Sepúlveda, 2007) and subsequent reproductive effects. Exposure to EDSs, e.g., have produced abnormal gonadal morphology, delayed sexual development and altered fish behaviour (Nash *et al*., 2004; Goksoyr, 2006; Della Seta *et al*., 2008), consequently affecting individuals and their progeny (Damstra *et al*., 2002).

EDSs can be introduced into aquatic environments via industrial wastes, sewage treatment plant (STP) effluents, agricultural runoff, and oil spills (Desbrow *et al.*, 1998; Ternes *et al.*, 1999; Rocha-Monteiro *et al.*, 2000). They can persist in the water for hours or even days (Jürgens *et al.*, 2002; Palace *et al*., 2006), and in locations far away from the discharge point (Harries *et al*., 1997).

1.2. Coastal environments and mummichog (*Fundulus heteroclitus*)

The east coast of North America contains large numbers of estuaries (Day *et al*., 1989a). Estuaries are one of the most productive types of ecosystems and they have unique geomorphologic and hydrologic characteristics (Day *et al*., 1989b). Estuaries have been modified by urbanization as coastal industries and municipalities discharge

increasing amounts of treated and untreated wastes into these aquatic environments. This contamination has the potential to affect different fish species since many migrate to estuaries to spawn (anadromous) and others live there permanently (Able *et al.*, 2005). The mummichog (*Fundulus heteroclitus*) is the numerically-dominant, small-bodied fish species present in salt marshes along the east coast of Canada and the U.S.A. This species is mostly sedentary, having small home ranges (Lotrich, 1975). They are adapted to the characteristic fluctuations of estuarine ecosystems and are able to survive salinities from 0.0 to 120.3 ppt (parts per thousand) and temperatures from -1.5ºC to 36.3ºC (Kneib, 1986). Spawning takes place in salt marshes, in the intertidal zone. Mummichog have a key role in the estuarine food network, since they are both predator and prey (Kneib, 1986). They have been used as a bioindicator species in numerous field studies to understand the potential impacts of contaminants (LeBlanc *et al.*, 1997; Fournier *et al.*, 1998; Couillard & Nellis, 1999). Their size, ability to breed in captivity, and resilience to factors such as variations in salinity, dissolved oxygen and temperature, make mummichog easy to maintain in laboratories and artificial streams (Burnett *et al*., 2007). In addition, this species has shown sensitivity to EDSs both in the laboratory (MacLatchy *et al.*, 2003; Peters *et al.,* 2007; 2010) and in artificial streams (Dubé *et al.*, 2002). Selection of this species as a fish model for laboratory and field studies to assess the effects of EDSs on estuarine and marine environments has been supported by numerous authors (Burnett *et al*., 2007; Bosker *et al.*, 2010; Greytak *et al.*, 2010; Lister *et al*., 2011).

1.3. 17α-Ethinylestradiol (EE_2): a model EDS

17α-Ethinylestradiol (EE_2) (Figure 1.1) is considered to be a representative EDS because it is a powerful estrogen agonist (Segner *et al*., 2003). It competes with the naturally-produced 17β-estradiol (E_2) (Figure 1.1) for the estrogen receptor (ER) (Pelissero *et al*., 1993, MacLatchy *et al.*, 2003). EE_2 has greater affinity for the ER than E_2 (Segner *et al.*, 2003; Denslow & Sepúlveda, 2007), although its effects depend on the relative circulating levels of E_2 in the fish (Kime *et al*., 1999). EE_2 is the principal pharmaceutical component of birth control pills and hormone replacement therapy, and is not broken down by sewage treatment processes (Desbrow *et al.*, 1998; Ternes *et al.*, 1999; Della Seta *et al*., 2008). Therefore, it enters aquatic environments in high concentrations through STP effluents. Concentrations of EE_2 present in British and Canadian STP effluents are usually between 0.2 and 7 ng/L EE_2 (Desbrow *et al*., 1998), although levels as high as 42 ng/L have been documented (Ternes *et al*., 1999).

EE_2 effects on fish reproduction in laboratory studies include a range of dysfunctions such as development of ovotestes in male Japanese medaka (*Oryzia lapites*; Balch *et al.*, 2004), reduced fertilization in mummichog (Peters *et al.*, 2007; 2010), and decreased egg production in zebrafish (*Danio rerio*; Nash *et al.*, 2004). In a whole-lake experiment, Kidd *et al.* (2007) found that exposure of a population of fathead minnow (*Pimephales promelas*) to EE_2 for two years caused feminization in males, a decreased rate of reproduction in the population, and reduced population fitness.

1.4. Fish reproductive endocrinology overview

Reproductive function in vertebrate organisms is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. Each level of the HPG axis synthesizes and releases a hormone or number of hormones. The hypothalamus produces gonadotropin releasing hormone (GnRH), which is the primary regulator of the reproductive system (Gore, 2002). In most animals, GnRH is released into the portal system through which it reaches the pituitary gland. However, in fish, GnRH neuroterminals project directly into the anterior pituitary gland and GnRH is released in the extracellular space (Gore, 2002). Consequently, the anterior pituitary secretes the gonadotropins lutenizing hormone (LH) and follicle stimulating hormone (FSH). These gonadotropins bind to their receptors in the ovaries and testes, to regulate folliculogenesis in females, spermatogenesis in males, and steroid biosynthesis in both sexes (Gore, 2002). The sex hormones such as E_2 and testosterone (T), released into the blood stream, act at their respective receptors in different tissues including the brain and the pituitary gland, where they produce feedback effects on the HPG (Gore, 2002; Hawkins *et al.*, 2005).

Interaction of E_2 and other steroids, including EDSs, occurs at target cells via two distinct mechanisms, one genomic and the other non-genomic (Legler *et al.*, 2002; Filby *et al.*, 2007; Vasudevan & Pfaff, 2008). The genomic actions are those where binding to ER results in regulation of transcription of steroidogenic genes (Filby *et al.*, 2007). The non-genomic response has been identified as a rapid increase (seconds or minutes) in cyclic AMP (cAMP), and is initiated at the plasma membrane by an interaction with ERs (Thomas & Doughty, 2004; Vasudevan & Pfaff, 2008).

1.5. Steroidogenesis

In response to the pituitary gonadotropins FSH and LH, cells in a variety of tissues (including the ovary, testis and adrenal or interrenal glands) from many animal species secrete steroids such as progestins, estrogens and androgens in a process termed steroidogenesis (Ings & Van der Kraak, 2006). Regardless of the cell type, steroids are synthesized from a common precursor substrate, cholesterol (Figure 1.1) (Veldhuis *et al.*, 1985). Cholesterol can be derived from cholesterol esters stored in intracellular lipid droplets, through *de novo* synthesis inside the cell, and from uptake of cholesterol contained in low density lipoproteins from the plasma (Miller, 1987). Once inside the cell, cholesterol is principally transported across the cytoplasm to the outer membrane of the mitochondria by specific carriers (Miller, 2007) and on a smaller scale is trafficked between vesicles and the plasma membrane (Maxfield & Wüstner, 2002; Soccio & Breslow, 2004; Miller, 2007). Steroidogenesis starts when cholesterol is transported from the outer to the inner membrane of mitochondria within steroidogenic cells (Leusch, 2001) (Figure 1.2). This process is presumed to be the rate-limiting step in mammalian steroid synthesis, and a protein called steroidogenic acute regulatory protein (StAR) is at least partially responsible for the cholesterol transport (Clark & Stocco, 1997; Arukwe, 2008). Within the mitochondria, cholesterol is enzymatically cleaved to form the first intermediate steroid, pregnenolone (Figure 1.1), by an enzyme called P450 side-chain cleavage (P450scc). P450scc is part of the cholesterol side-chain cleavage system (CSCC) that resides in the inner membrane of mitochondria. Once pregnenolone is synthesized, it may be metabolized to progesterone (Figure 1.1) by 3β-hydroxysteroid

dehydrogenase (3β-HSD), or can be transformed to androgens (Figure 1.1) by cytochrome P450 17α-hydroxylase/17,20 lyase (P45017α). Testosterone (T) (Figure 1.1), one of the main vertebrate androgens, is converted into E_2 by cytochrome P450 aromatase (P450 arom; Arukwe 2008; Leusch, 2001) (Figure 1.2). In male fish T is converted into 11-ketotestoterone (11-KT) by 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase (11β-HSD) and this androgen is known to be the principal androgen in teleost fish (Figure 1.2; Kime, 1993). These reproductive steroids have important roles in vertebrates such as regulation of reproductive function and secondary sex characteristics in females, and fertility and secondary sex characteristic in males (Stocco, 2001).

In steroidogenic cells, pituitary gonadotrophins regulate synthesis of different steroids via regulation of expression and activity of the steroidogenic enzymes and proteins. Once gonadotrophin binds to its receptors on the cell membrane, the cAMP second messenger system is activated (Taskén & Aandahl, 2004). As a consequence, cAMP is synthesized and activates protein kinase A (PKA), generating two types of responses: acute and chronic. In the acute response, PKA phosphorylates and activates cholesteryl esterase. These esterases hydrolyze cholesteryl esters contained in lipid droplets to free cholesterol (Azhar *et al.*, 2003). Although there is a debate on how it happens, several carriers such as sterol carrier protein 2 (SCP_2) or StAR-D4, StAR-D5, StAR-D6 appear to be mainly responsible for the transport of cholesterol from the cytosol to the outer membrane of the mitochondria (Seedorf *et al.*, 2000; Azhar *et al.*, 2003; Miller, 2007). In the chronic response, PKA is involved in the activation of the expression of specific genes required for estrogen, androgen and progesterone synthesis in steroidogenic cells, including genes responsible for the biosynthesis of the involved

proteins and enzymes: StAR, P450scc, 3β-HSD and P450arom (Taskén & Aandahl, 2004). Cyclic AMP can also be negatively regulated inside the cells by another group of proteins called phosphodiesterases (PDEs) that degrade cAMP when it is no longer needed, consequently limiting gene expression of the enzymes involved in steroidogenesis (Jin *et al*.,1999).

1.6 Effects of environmental factors on steroidogenesis

Environmental factors such as salinity and temperature have been shown to affect fish reproductive endocrine status. For example, waigieu sea perch (*Psammoperca waigiensis*) had a gradual increase of female plasma E_2 with increasing salinity during pre-spawning and spawning periods for fish kept at 10, 20 and 32 ppt. Plasma T also differed among the salinity groups. Plasma T was higher in the pre-spawning period when females were kept in 32 ppt compared to females kept at 10 and 20 ppt and significantly lower after spawning than the 20 and 10 ppt groups (Quoc Pham *et al.*, 2010). Male black bream (*Acanthopagrus butcheri)* kept through a natural reproductive cycle at 35 ppt had higher plasma levels of 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 11-KT than those kept at 5 ppt and 20 ppt (Haddy & Pankhurst, 2000).

A variation in temperature also produces effects on fish reproductive endocrinology. Wolffish (*Anarhichas lupus L.*) had a delay of four weeks in the normal reproductive cycle, resulting in delayed ovulation, due to exposure to elevated temperatures (Tveiten & Johnsen, 2001). During summer, female Atlantic salmon (*Salmo salar L.*) kept at 22ºC had lower E_2 and higher T than females kept at 14 or 18ºC, with the

lowest E_2 levels at 18ºC (King *et al.*, 2003). Elevated temperatures affected pejerrey (*Odontesthes bonariensis*) reproductive endocrine function as T in males and E_2 in females were significantly decreased with increased temperature (Soria *et al*., 2008).

1.7. Effects of EE_2 on steroidogenesis

The most common estrogenic compounds found in aquatic environments close to STPs are EE_2 and E_2 (Desbrow *et al.*, 1998). EE_2 mimics the biologically-synthesized E_2 and produces strong effects on steroidogenesis in different fish species. For example, sea bream (*Sparus aurata*) exposed to 25 mg/kg of EE_2 in their diet, followed by *in vitro* incubations of their gonads with androstenedione as a precursor, showed increased production of E_2 (37 to 112 d of exposure) and T (112 d of exposure) than the control group (Condeça & Canario, 1999). In mummichog, waterborne exposure to concentrations of 1-100 ng/L of EE_2 altered gonadal steroid production in females and caused completion of spawning cycles earlier compared to females exposed to lower EE_2 concentrations (0-0.1 ng/L) (MacLatchy *et al.*, 2003; Peters *et al*., 2007). *In vitro* incubation of Atlantic croaker (*Micropogonias undulatus*) testes with 36.7 μM of E_2 in the presence of the precursors pregnenolone, 17-hydroxyprogesterone, and androstenedione inhibited 17α-hydroxylase, 17, 20-desmolase (CYP17A1), or 17β-dehydrogenase (17β-HSD) activity (Loomis & Thomas, 2000). Sea bream exposed for 14 d to 2 and 15 mg/kg of E_2 in the diet resulted in a significant increase in enzymatic activity of 3β-HSD during the first week, whereas 11β-HSD and 17β-HSD were substantially lower than the control group in the highest concentration of E_2. Exposure to

E_2 also caused greater T synthesis than the control group in the 14-d exposure (Condeça & Canario, 2001). When rainbow trout (*Oncorhynchus mykiss*) males were treated with E_2 an inhibition of the activity of 3β-HSD and 11β-hydroxylase occurred (Baron *et al.*, 2005; Govoroun *et al.*, 2001). Male mummichog exposed to 250 ng/L of EE_2 showed a significant reduction in the production of T in basal and 25-OH cholesterol-stimulated gonadal incubations, demonstrating that while StAR and P450scc may be affected by EE_2, an effect downstream in the steroidogenic pathway could also be present (Hogan *et al.*, 2010)

EE_2 has also been shown to alter gene expression in various fish species. For instance, rainbow trout males exposed for 16 d to EE_2 showed an inhibition of the expression of the mRNA for 3β-HSD and P45017α after the first day, and an inhibition of P450scc and 11β-hydroxylase after the eighth day (Baron *et al.*, 2005). Fathead minnow treated with EE_2 showed a significant increase in the mRNA expression of aromatase A (CYP19a) in male, and aromatase B (CYP19b) in female gonads, a significant reduction in 17β-HSD and StAR mRNA, and an increase in 17β-HSD in the mRNA extracted from female gonads (Filby *et al.,* 2007). StAR and major steroidogenic enzyme genes such as CYP19a and 3β-HSD were significantly decreased in one-year-old females of the same species exposed to E_2 (Nakamura *et al.*, 2009).

1.8. Gaps in knowledge

To date, most of the research done on the effects of estrogenic compounds on fish steroidogenesis has been done on stenohaline freshwater species (e.g., zebrafish and

fathead minnow). Understanding how estrogenic compounds affect reproductive endocrine status necessarily includes determining effects on production and circulating levels of the terminal steroid concentrations as the terminal steroids regulate development, growth, and reproduction by interacting with receptors in other organs, including the brain, liver and gonads. However, less work has been done to understand the effects of estrogenic compounds on steroidogenesis and circulating steroid levels in saltwater or estuarine species. Although inferences can be made from studies on freshwater species, because environmental factors (e.g., salinity and temperature) affect reproductive endocrine status in fish, more focused studies in estuarine fish are warranted. This work is unique in that it is the first study to consider the possible interaction between varying environmental conditions and the effects of EE_2 on steroidogenesis.

1.9. Integrative approach

This project used an integrative approach to elucidate the potential interaction of environmental parameters with EE_2, a model endocrine disruptor, and the effects of EE_2 on physiological function at the biochemical and molecular levels.

Analyzing the effects of EE_2 on the circulating steroid levels allowed us to determine how the general physiology of mummichog, a model estuarine species, is affected. Investigating more deeply by determining EE_2 effects on gonadal steroidogenesis let us postulate a mechanism by which circulating steroid levels are altered by EE_2. Finally, working at the molecular level allowed us to begin determining

whether the effect of EE_2 on steroidogenesis is via genomic and/or non genomic mechanisms. The approach of studying multiple levels of biological organization (whole organism to tissue to molecular level) combined with effects of potentially interactive environmental stressors (salinity and temperature, and a contaminant), is clearly highly integrative.

1.10. Objective and hypotheses

The objective of this study is to evaluate if variations in salinity or temperature conditions of EE_2-exposed mummichog modify the effect on whole-organism reproductive endocrine status and gonadal steroid biosynthesis.

The null hypotheses are:

- Level 1: Organ weight and somatic indices

  - Ho_{1A}: EE_2 does not affect organ weight and somatic indices
  - Ho_{1B}: Salinity (or temperature) does not affect organ weight and somatic indices
  - Ho_{1C}: There is no interaction of EE_2 and salinity (or temperature) on organ weight and somatic indices

- Level 2: Circulating Steroids

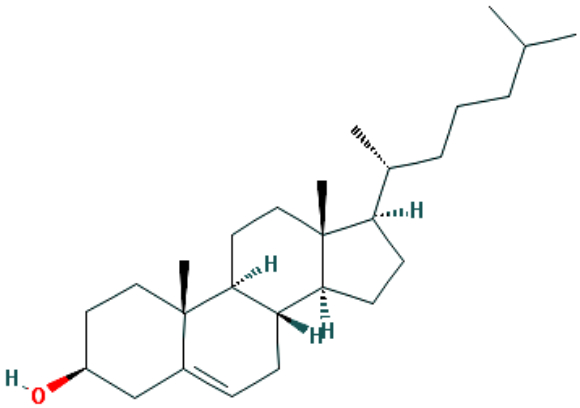
  - Ho_{2A}: EE_2 does not affect circulating steroid levels
  - Ho_{2B}: Salinity (or temperature) does not affect circulating steroid levels
  - Ho_{2C}: There is no interaction of EE_2 and salinity (or temperature) on circulating steroid levels

- Level 3: Gonadal Biosynthesis

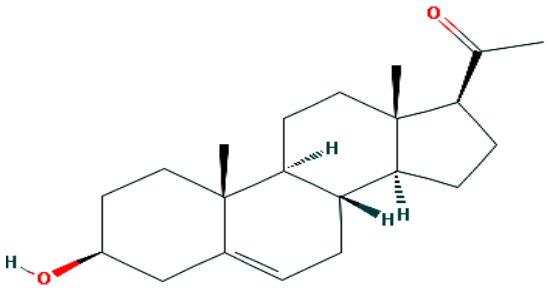
  - Ho_{3A}: EE_2 does not affect biosynthesis of gonadal terminal steroids
  - Ho_{3B}: Salinity (or temperature) does not affect biosynthesis of gonadal terminal steroids
  - Ho_{3C}: There is no interaction of EE_2 and salinity (or temperature) on biosynthesis of gonadal terminal steroids

- Level 4: Gene Expression

  - Ho_4: EE_2 does not affect P450 aromatase A gene expression



Cholesterol



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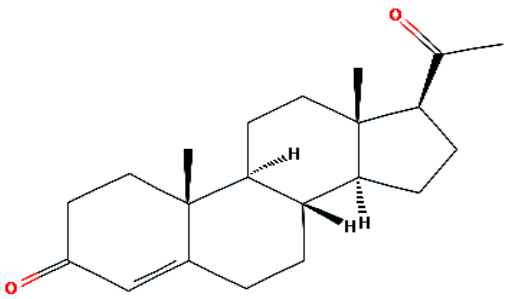

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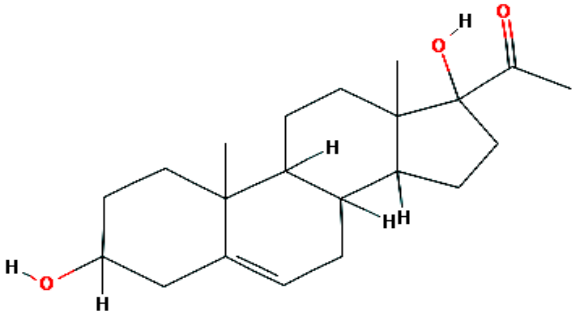

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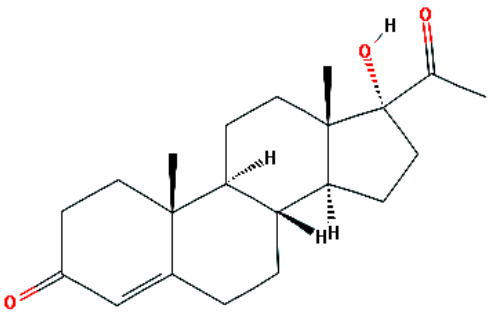

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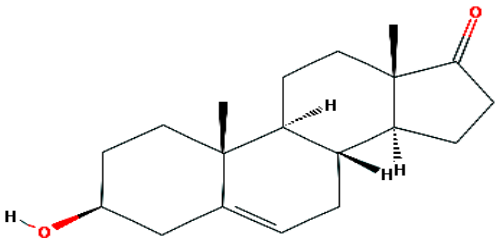

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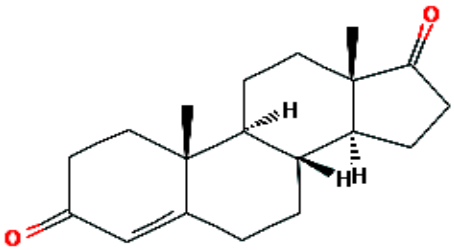

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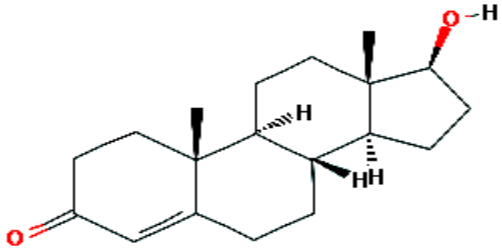
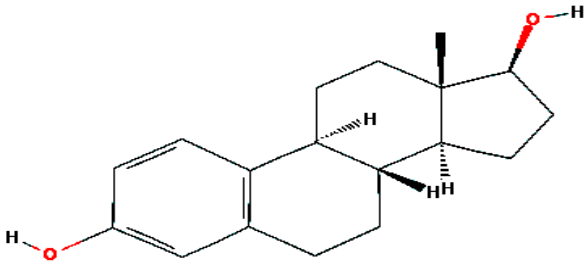
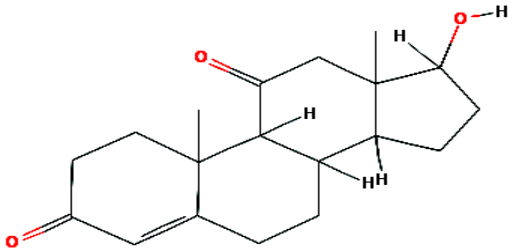


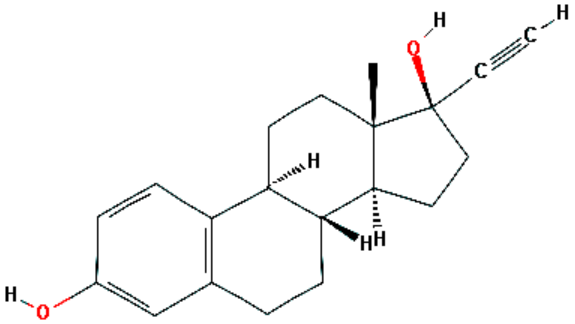
Testosterone



17β-Estradiol (E_2)



11-Ketotestosterone



17α-Ethinylestradiol (EE_2)

Figure 1.1: Chemical structures of the intermediate and final products of the gonadal steroidogenic pathway in fish and the synthetic 17α-ethinylestradiol. http://pubchem.ncbi.nlm.nih.gov/

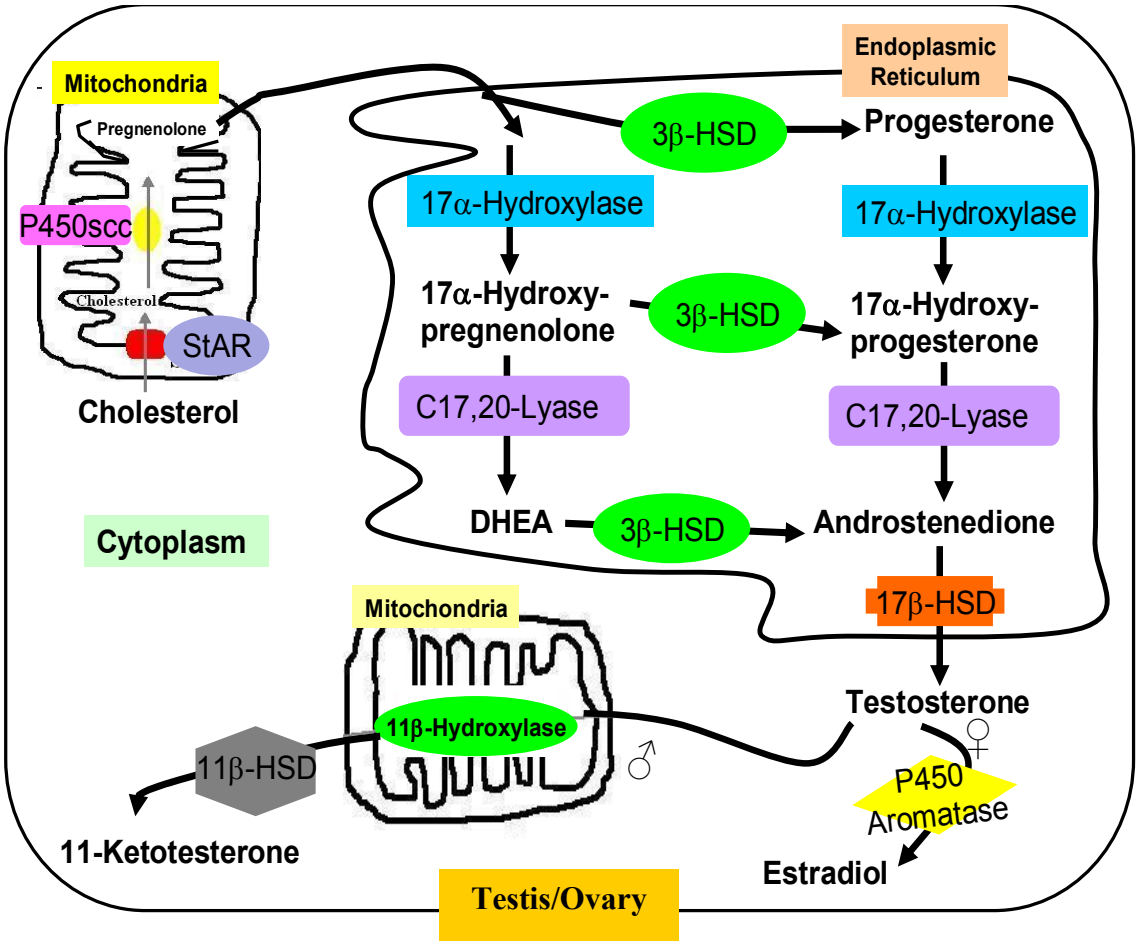


Figure 1.2: Gonadal steroidogenesis in fish. The protein and enzymes involved in the steroidogenic pathways are: steroidogenic acute regulatory protein (StAR); P450 side-chain cleavage (P450scc); 3β-hydroxysteroid dehydrogenase (3β-HSD); 17α-hydroxylase; C17,20-lyase; 17β-hydroxysteroid dehydrogenase (17β-HSD); P450 aromatase; 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase (11β-HSD). Dehydroepiandrosterone (DHEA) is an intermediate steroid in the pathway. Modified from McMaster *et al.*, 1995 and Leusch, 2001.

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Chapter 2

Research Paper

2.1 Abstract

Exposure to 17α-ethinylestradiol (EE_2), a synthetic estrogen, has previously been shown to decrease reproductive endocrine status and egg production in northern mummichog (*Fundulus heteroclitus macrolepidotus*). The objective of this study was to evaluate if variations in salinity or temperature conditions of EE_2-exposed mummichog modify the effect on whole-organism reproductive endocrine status and gonadal steroidogenesis. Mummichog were exposed in vivo for 14 days to 0, 50 and 250 ng/L EE_2 in 0, 16 and 32 ppt salinity at 18ºC and to 0 and 250 ng/L EE_2 at 10, 18 and 26°C at 16 ppt. In the salinity exposure, 250 ng/L EE_2-exposed females had significantly reduced 17β-estradiol (E_2) levels. Increased temperature triggered gonadal growth in both sexes and increased plasma E_2 and E_2 production and decreased 11-KT (11-ketotestosterone) production. EE_2 counteracted the effect of temperature by depressing gonadal growth in males. In both exposures, EE_2 effects on testosterone (T) production were variable. The use of steroidogenic precursors (25-OH cholesterol, and/or pregnenolone and/or testosterone) in the *in vitro* gonadal incubations indicated decreased E_2 production in females and 11-KT production in males were predominately due to suppression of the terminal conversion step between T and E_2 or 11-KT. Ovarian cyp19a gene expression at 16 ppt and 18ºC was not affected by 250 ng/L EE_2 (the only treatment combinations tested). Overall, temperature is a factor regulating northern mummichog reproduction; EE_2 overrides its effects and disrupts the terminal step of steroidogenesis. Our results should be considered in designing future estuarine fish bioassays and in understanding effects of estrogenic endocrine disruptors in estuaries.

2.2 Introduction

A significant body of literature has been developed that demonstrates the effects of environmental endocrine disrupting substances (EDSs) on fish reproductive physiology (Damstra *et al.*, 2002, Ankley & Johnson, 2004; Tarrant *et al.*, 2005; Hecker *et al.*, 2006; Sharpe *et al.*, 2007; Lister *et al.*, 2011). EDSs are introduced into aquatic environments through sewage treatment plant (STP) effluents (Desbrow *et al.*, 1998; Ternes *et al.*, 1999), agricultural run off (Colucci & Top., 2001; Burnison *et al*., 2003), industrial waste (Castillo & Barceló, 1997; Hewitt *et al*. 2008) and oil spills (Rocha-Monteiro *et al.*, 2000; Martin-Skilton *et al.*, 2008). Among EDSs, estrogenic compounds are of major interest because they alter reproductive behaviour (Balch *et al.*, 2004; Larsen *et al.*, 2008; Salierno & Kane, 2009), fecundity (Nash *et al.*, 2004; Thorpe *et al.*, 2009) and the biosynthesis of terminal steroids (Loomis & Thomas, 2000; Baron *et al.*, 2005).

One of the most common estrogenic compounds found in the aquatic environment near STP discharging points is 17α-ethinylestradiol (EE_2; Desbrow *et al.*, 1998). EE_2 is a synthetic estrogen used in birth control pills and hormone replacement therapy. It is not fully removed by wastewater treatment and thus persists in treated effluents (Kuch & Bullschmiter, 2001; Jürgens *et al*., 2002). EE_2 mimics the biologically-synthesized 17β-estradiol (E_2), exhibiting higher affinity for the estrogen receptor (ER) in many species (Condeça & Canario, 1999; Segner *et al.*, 2003; Hogan *et al.*, 2010). This interaction triggers genomic and non-genomic responses, potentially affecting fish reproductive endocrine systems (Legler *et al.*, 2002; Thomas & Doughty, 2004; Filby *et al.*, 2007; Vasudevan & Pfaff, 2008).

The effects of EE_2 on circulating steroid levels have been well documented in fish. After exposure, E_2 was depressed in male goldfish (*Carassius auratus*) exposed to 29.6 and 296 ng/L EE_2 for 15 d (Martinyuk *et al.*, 2006) and in mummichog (*Fundulus heteroclitus*) exposed to 500 ng/L EE_2 for 7 d (MacLatchy *et al.*, 2003) and to 100 ng/L-EE_2 for 21 d and 10 and 100 ng/L EE_2 for 28 d (Peters *et al.*, 2007). Also, testosterone (T) was depressed in male goldfish (Martinyuk *et al.*, 2006) and female mummichog exposed to 100 ng/L EE_2 for 21 d (Peters *et al.*, 2007). Decreased circulating 11-ketotestosterone (11-KT) occurred in male rainbow trout (*Oncorhynchus mykiss*) exposed to 100 ng/L of EE_2 for 62 d (Schultz *et al.*, 2003), as well as in mummichog exposed to 1, 10 and 100 ng/L EE_2 for 21 d (Peters *et al.*, 2007) and in juvenile turbot (*Psetta maxima*) when exposed to 3.5 and 75 ng/L EE_2 for 15 d (Labadie & Budzinski, 2006). EE_2 has also been shown to increase circulating 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) levels in male rainbow trout (Schultz *et al.*, 2003). Variations in response patterns may be due the timing of exposure in relation to the reproductive endocrine cycle and when the fish were sampled.

Gonadal incubations have been useful tools for elucidating effects and mechanisms of action of EDSs on steroidogenesis. After exposure to EE_2, there was a significant decrease in gonadal production of 11-KT in male juvenile turbot (3.5 and 75 ng/L EE_2 for 15 d, Labadie & Budzinski, 2006), and a significant depression of E_2 production in mummichog ovaries (500 ng/L EE_2 for 7 d, MacLatchy *et al*., 2003; 10 ng/L EE_2 for 28 d, Peters *et al.*, 2007). In addition, steroidogenic precursors used in gonadal incubations have helped to determine the mechanistic effects of EE_2. Androstenedione (Figure 1.2) used in sea bream (*Sparus aurata*) gonadal incubations

demonstrated that exposure *in vivo* to EE_2 (15 mg of EE_2/kg of diet for 14 weeks) produces an increase in T and E_2 production after 14 weeks of exposure. It was also demonstrated that the effect of EE_2 was downstream of the conversion of androstenedione to T (Condeça & Canario, 1999). Forskolin, which activates adenylate cyclase, thus increase levels of cellular cAMP, was used in female pearl dace (*Margariscus margarita*) gonadal incubations, and indicated that an increase in T and a decrease in E_2 was downstream of cAMP activation, when fish were previously exposed to EE_2 (4-8 ng/L; 21 weeks; Palace *et al.*, 2006). The addition of 25-OH cholesterol (analogue to cholesterol which can pass through the mitochondrial membrane) to male gonadal incubations demonstrated that exposure of mummichog to EE_2 (250 ng/L for 14 d) resulted in a reduction in T production downstream of cholesterol mobilization (Hogan *et al.*, 2010).

Fish living in estuaries experience daily fluctuations in salinity and temperature as well as potential exposure to estrogenic contaminants from coastal discharges. Both salinity and temperature affect fish steroidogenesis. An increase in salinity from 0 ppt to 32 ppt produced an increase in plasma E_2 in pre-spawning and spawning waigieu sea perch (*Psammoperca waigiensis*) females and an increase in T levels during the pre-spawning period (Quoc Pham *et al.*, 2010). Higher plasma 11-KT and 17,20βP were observed in black bream (*Acanthopagrus butcheri)* exposed to 35 ppt compared to 5 ppt salinity, when analyzed through a natural cycle (Haddy & Pankhurst, 2000). An increase in temperature from 12-15 to 18ºC caused a decrease in E_2 and T in female rainbow trout follicles (Pankhurst *et al.*, 1996). Similarly, in pejerrey (*Odontesthes bonaeriensis*) an

increase in water temperature from 19 to 27ºC caused a decrease in plasma T and E_2 (Soria *et al.*, 2008).

To date, the majority of studies on the effects of EDSs and their mechanisms of action have used standardized laboratory conditions (e.g., a fixed salinity and temperature). Studies on the interactions of environmental variables and EDSs on reproductive responses are warranted, both from the perspective of understanding the applicability of standardized conditions in fish reproductive tests, as well as determining the potential impact of changing environmental parameters on EDS responses in estuarine fish in the wild. Mummichog is an ideal species to investigate the effects of environmental conditions on responses to EDSs, as this species has shown reproductive endocrine sensitivity to EDSs, particularly to EE_2 (MacLatchy *et al.*, 2003; Peters *et al.,* 2007; 2010; Hogan *et al.,* 2010). Additionally, mummichog experience daily variations in salinity and temperature and are adapted to the characteristic fluctuations of estuarine ecosystems, surviving in salinities between 0.0 to 120.3 ppt and temperatures from -1.5ºC to 36.3ºC (Kneib, 1986; Burnett *et al*., 2007).

The objective of this study was to evaluate if variations in salinity or temperature modified the effect of EE_2 exposure on reproductive endocrine endpoints in mummichog. Male and female fish were exposed for 14 d to 50 and 250 ng/L of EE_2 and gonad size, plasma steroid levels, gonadal steroid production and ovarian aromatase expression were assessed. The results indicate that environmental conditions do cause modifications of EE_2 responses in mummichog.

2.3. Materials and Methods

2.3.1 Chemicals and supplies

17α-Ethinylestradiol (EE_2), testosterone (T) and 17β-estradiol (E_2) standards, 3-isobutyl-1-methylxanthine (IBMX) and the steroidogenic precursors (see details in 2.3.5) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Concentrated stocks of EE_2 were prepared in 100% ethanol at a concentration of 1000 ng/L and 5000 ng/L of EE_2 and stored in glass bottles at -20ºC. All remaining supplies were purchased from Fisher Scientific (Ottawa, ON) or as otherwise described in the methodology.

2.3.2 Animal holding

Adult mummichog were collected from the Shediac area, New Brunswick, Canada (46º 16'N; 64º 30'W). Fish were kept at the University of New Brunswick, Saint John, NB at 16 ppt of salinity in 250L fiberglass tanks, at room temperature, 16 h light:8 h dark and $\geq$80% dissolved oxygen (DO). Fish were fed *ad libitum* daily with commercial pellets (Corey Feed Mills, Fredericton, NB) and mortality was less than 5%. Prior to the exposures, temperature and salinity were adjusted by increasing or decreasing daily temperature and salinity by 2ºC and/or 3 ppt progressively until the desired temperature or salinity was reached. The source of fresh water was dechlorinated Saint John city water, while sea water was filtered from the Bay of Fundy (32 ppt). Brackish water (16 ppt) was made by combining sea and fresh water.

2.3.3 Exposures

For the salinity exposure, three females and three males were randomly chosen from the stock tanks and put into 20L glass tanks (four replicates per treatment) at salinities of 0 (fresh water), 16 (brackish water) and 32 (full sea water) ppt at 18ºC for one week prior to start of the exposures. Fish were exposed for 14 d (August 24 to September 7, 2010) to EE_2 under static conditions, with daily water renewal. EE_2 treatments were 0, 50 and 250 ng/L (nominal) delivered in 1 mL of ethanol following the daily water exchange. Photoperiod, DO and feeding regimen were maintained as during the holding period.

For the temperature exposure, the experimental set up was as previously described for the salinity exposure. However, temperatures were set at 10, 18 and 26ºC, with salinity maintained at 16ppt in all treatments, and EE_2 concentrations were 0 and 250 ng/L.

2.3.4 Sampling

On day 14, all fish were randomly (by tank) anaesthetized with buffered 0.05% tricaine methane sulfonate (Syndel Laboratories, Vancouver, BC, Canada), bled from the caudal vasculature using heparinized 26 3/8 gauge needles on 1 mL syringes, and killed by spinal severance (in accordance with the guidelines of the Canadian Council on Animal Care and the Local Animal Care Committee at the University of New Brunswick). Fish were weighed (±0.01g) and measured for length (±mm), and the gonads

and liver were dissected and weighed (±0.001g). Gonad weight relative to body weight (expressed as gonadal somatic index [GSI; 100×gonad wt/body wt]) and liver weight relative to body weight (expressed as liver somatic index [LSI: 100×liver wt/body wt]) were calculated. The blood was centrifuged (3000×*g* at 4°C, Sorvall Legend RT Centrifuge; Thermo Scientific, Nepean, ON) for 20 min to isolate plasma (MacLatchy *et al.,* 2005). Plasma was stored at -20°C for later steroid measurement by radioimmunoassay (RIA) or enzymatic immunoassay (EIA). The dissected gonads were placed into fresh Medium 199, prepared according to McMaster *et al.* (1995) (pH = 7.4) and supplemented as suggested by MacLatchy *et al.* (2005) with IBMX at a final concentration of 1 mM at the start of the incubation.

2.3.5 *In vitro* incubation protocol

The incubation protocol was based on McMaster *et al.* (1995) and MacLatchy *et al.* (2005). A minor variation to the protocol was implemented as follows: one piece of ovary/testes from each fish was pooled with tissue from the other fish of the same sex within the same treatment tank. The combined tissue samples were placed in the same treatment well in a 24-well incubation plate (18-23 mg of tissue per well). There were four replicates per treatment. Gonads were incubated for a period of 18 h at 18ºC in both basal medium and media with steroidogenic precursors. The basal medium was composed of Medium 199 and IBMX. Steroidogenic precursor media were prepared with basal medium plus the addition of the precursors. The precursors and their concentrations were chosen according to McMaster *et al.* (1995) and were as follows: in the ovarian

incubation 25-OH cholesterol (5 μg/mL) to identify effects downstream of cholesterol mobilization into mitochondria; pregnenolone (100 ng/mL) to identify effects downstream of pregnenolone/P450scc; and testosterone (100 ng/mL) to identify effects downstream of testosterone/aromatase. The testes were only incubated with 25-OH cholesterol (5 μg/mL) due to limited tissue availability. After 18 h of incubation, the medium from each well was drawn into 1.5 mL eppendorf tubes. The tissue was discarded and the tubes were kept frozen at -20°C until RIA or EIA analysis.

2.3.6 Radioimmunoassay and enzymatic immunoassay

Plasma samples were prepared for RIA and EIA by extracting the steroids and reconstituting them in a phosgel assay buffer (MacLatchy *et al.*, 2005). The extraction process was repeated three times prior to reconstitution. RIA (MacLatchy *et al.*, 2005) was used to determine the T and E_2 concentrations in female plasma and gonadal incubation media and T in male plasma and gonadal incubation media. Antibodies for T and E_2 were purchased from Medicorp (Montreal, QC, Canada) and radiolabelled T and E_2 from Perkin-Elmer (Waltham, MA, USA). EIA kits were used to measure 11-KT in male plasma and male gonadal incubation media. Kits were obtained from Cayman Chemical Co. (Ann Arbour, MI, USA) and the manufacturer's protocol (http://www.caymanchem.com/) was followed with the exception that 25 μL of sample and 25 μL of EIA buffer were used instead of 50 μL of sample and buffer. EIA plates were read in a Spectramax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA) Model at 412 nm after 60, 90 and 120 min to determine the optimum incubation time. All

inter- and intra-assay variations were within acceptable limits (<10% for intra-assay and <15% for inter-assay).

2.3.7 Ovarian P450 Aromatase A (cyp19a) gene expression

Ovarian P450 aromatase A gene expression was analyzed in ovarian tissue of fish held at 16 ppt and in 18°C, at 0, 50 and 250 ng/L EE_2, to investigate a possible mechanism of action of EE_2. The analysis methods are described as follows.

2.3.7.1. RNA extraction and quantification

Total RNA extraction, quantification and reverse transcription reactions were conducted similarly to Ings & Van Der Kraak (2006) and Nelson & Van Der Kraak (2010). Total RNA was extracted from ovarian follicles using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Burlington, ON). Following the extraction, total RNA was pelleted by centrifugation, rinsed with 75% ethanol, then reconstituted in 20-30 μL RNase-free water (Invitrogen) and incubated at 60ºC for 10 min to fully dissolve the RNA pellet. Absorbance of RNA was measured at wavelengths of 260 nm and 280 nm using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA). All samples had an A260/A280 ratio between 1.8 and 2.2.

2.3.7.2. Reverse transcription

RNA was diluted in RNase-free water to a concentration of 1 µg/µL and 2 µg was used in reverse transcription (RT) reactions to generate cDNA. Each sample was treated with 1 µL DNase 1 (AMP-D1, Sigma-Aldrich) according to the manufacturer's protocol. Random primers (0.01 ng, Promega, Madison, WI, USA) were added to each sample then incubated for 5 min at 70°C. A cocktail consisting of 5x RT buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, Invitrogen), RNase-free water, dNTPs (0.5 mM, Roche Molecular Biochemicals, Laval, QC), DTT (10 mM, Invitrogen) and M-MLV reverse transcriptase (200 U, Invitrogen) was added to each sample to obtain a final volume of 25 µL. The reverse transcription reactions were completed in an Eppendorf Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) for 1 h at 37ºC, followed by 5 min at 90ºC, then diluted 5-fold and stored at -20ºC until qPCR amplification.

2.3.7.3. Quantitative PCR

cDNA was amplified using the SYBR method and StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA). The reaction contained 3.75 µL of cDNA template, 7.5 µl PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD, USA), and 1.875 µL of forward and reverse primers (1.6 µM, Sigma-Aldrich). Primer sequences are shown in Table 2.1.

The cycle consisted of 5 min at 95°C, then 40 cycles of 1 s at 95°C, followed by 30 s at 60°C. The expression levels of elongation factor-1α (*ef1α*) remained unchanged

across treatments and were used as an endogenous control gene to normalize the gene of interest. A four-point standard curve was run for each gene in order to quantify gene expression.

Table 2.1: Primer sequences analyzed (FWD means forward primer and REV means reverse primer).

Gene		Sequence 5'-3'	Accession Number
Aromatase A (cyp19a)	FWD	TGCCCCTCGACGAGAAAG	AY713118.1
	REV	GTAGATGTCGGGTTTGATCAGCA	
Elongation factor-1α (ef1a)	FWD	ACCAGAAAGTACTACGTGACCATC	AY430091
	REV	TCAGCCTGGAGGTACCG	

2.3.8. Statistics

Statistical analysis was conducted using SPSS 17.0 (IBM Canada Ltd., Markham, ON) or Sigma Plot (Systat Software INC, Chicago, IL, USA). Two-way analysis of variance (ANOVA) was performed to assess the effect of salinity or temperature (factor 1) and EE_2 (factor 2) on the measured endpoints. The effect of the two main factors on body weight, liver weight, gonad weight, plasma steroid levels and gonadal steroid levels were analyzed by two-way ANOVA. When there was no factor interaction or no effect of salinity or temperature, data were pooled to analyze the effect of EE_2 by one- or two-way ANOVA, as required. When interaction or a salinity/temperature effect on the basal incubation media was present, the effect of EE_2 on steroidogenesis was analyzed separately in each salinity or temperature. Liver weight and gonad weight relative to body weight were analyzed by two-way analysis of covariance (ANCOVA) with body weight

as covariant. Assumptions of normality and homogeneity of variance were checked using Shapiro-Wilk's W test and Levene's test, respectively. If there was no normal distribution of the data, log transformations were conducted and normality was tested for a second time. In case of normality failure after the statistical transformations in the two-way ANOVA, due to the robustness of the ANOVA test, the post hoc tests were analyzed without considering the normality failure of the ANOVA. For the one-way ANOVA, the non-parametric Kruskal-Wallis test was performed if normality was not met following log transformation. Tests in which post hoc tests were used even if there was a failure of normality of the two-way ANOVA are indicated as NP and tests in which the Kruskal-Wallis test was used as the non parametric alternate to the one-way ANOVA are indicated as KW in section 2.4. The parametric Tukey's test was performed as post hoc analysis, for pair wise comparisons. In all cases, an alpha value of 0.05 was chosen.

Gene expression data were analyzed using one-way ANOVA. Normality and homogeneity of variance were tested as previously described. A Dunnett's test was conducted as a post hoc test. Data were log transformed since they did not initially meet the normality assumption.

2.4. Results

2.4.1. Effects of EE_2 under differing saline conditions

2.4.1.1. Organ weights and somatic indices

There was no interaction between salinity and EE_2 ($p=0.240$), nor effect of salinity ($p=0.163$) or EE_2 ($p=0.536$) on female weight (Table 2.2). In males, there was no interaction between salinity and EE_2 ($p=0.133$), but there was a significant effect of salinity on male weight. Body weight of fish in 32 ppt was higher than in 16 ($p<0.001$) or 0 ($p<0.001$) ppt. There was no effect of EE_2 on male weight ($p=0.406$). There was no significant effect of salinity ($p=0.086$) nor EE_2 ($p=0.717$) on female liver weight. There was no interaction ($p=0.291$), but there was a significant increase for male liver weight with the increase in salinity ($p<0.001$). There was no significant effect of EE_2 on male liver weight ($p=0.515$; Table 2.2). No interaction between salinity and EE_2 ($p=0.820$), nor significant effect of salinity ($p=0.695$), nor effect of EE_2 ($p=0.647$) was found when female gonad weight was analyzed. In addition, there was no interaction between the two main factors for male gonad weight ($p=0.816$), but there was a significant increase in male gonad weight with the increase in salinity ($p<0.001$). There was no effect of EE_2 on male gonad weight ($p=0.076$; Table 2.2).

For female LSI, there was interaction between salinity and EE_2 ($p=0.044$), but the post hoc tests were not able to distinguish between treatments. In males, there was no interaction between the two main factors ($p=0.654$, data not shown), but there was a

significant effect of salinity on LSI ($p<0.001$). Males in 32 ppt had higher LSI than males in 16 ppt. There was no effect of EE_2 on LSI on males ($p=0.480$; Table 2.2).

In females, there was no interaction between the two main factors ($p=0.940$) or effect of salinity ($p=0.218$) on female GSI. There was no effect of EE_2 on female GSI ($p=0.269$). In males there was no interaction between salinity and EE_2 ($p=0.813$), but there was a significant effect of salinity on male GSI ($p<0.001$) as in 32 ppt male GSI was higher than in 0 and 16 ppt. There was no effect of EE_2 on male GSI ($p=0.269$; Table 2.2).

2.4.1.2. Plasma steroid analysis in females

There was no significant interaction of salinity and EE_2 on female plasma T or E_2 ($p=0.601$, $p=0.186$; data not shown). Neither salinity ($p=0.387$) nor EE_2 ($p=0.837$) affected female plasma testosterone levels (Figure 2.1A). There was no effect of salinity on plasma E_2 levels ($p=0.902$; Figure 2.1B); however, 250 ng/L EE_2 caused a significant reduction of female plasma E_2 levels ($p=0.045$) at 16 ppt (Figure 2.1B; Table 2.3).

2.4.1.3. Gonadal steroid production in females

In the ovarian incubations, there was interaction between salinity and EE_2 when female T was analyzed ($p<0.001$; data not shown). At 0 ppt, there was a significant increase in T production in the basal medium of 250 ng/L EE_2-exposed fish ($p=0.036$; Figure 2.2A (NP)). There was no change in female basal T production ($p=0.649$; Figure

2.2B). There was a significant decrease in 25-OH cholesterol stimulated T incubations in the 250 compared to the 50 ng/L exposure ($p=0.044$; Figure 2.2B (NP)) in females exposed to EE_2 in 16 ppt. Females kept at 32 ppt had a significant reduction in the basal production of T at 50 ng/L and 250 ng/L of EE_2 ($p<0.001$; $p=0.005$, respectively; Figure 2.2C). There was no interaction between salinity and EE_2 on basal E_2 production ($p=0.436$; data not shown), and no effect of salinity on biosynthesis of E_2 in basal media ($p=0.680$; data not shown). When salinity data were combined, females exposed to 250 ng/L EE_2 showed a significant reduction in E_2 production in the basal ($p<0.001$), 25-OH cholesterol ($p=0.004$), pregnenolone ($p<0.001$) and testosterone ($p=0.029$) incubated ovaries (Figure 2.2D; Table 2.3).

2.4.1.4. Plasma steroid analysis in males

In males, there was no significant interaction between salinity and EE_2 ($p=0.152$; $p=0.475$; data not shown) and no significant effect of the salinity ($p=0.269$; Figure 2.1C; $p=0.678$; Figure 2.1D) on the plasma T and 11-KT concentrations, respectively. There was no effect of EE_2 ($p=0.584$; Figure 2.1C) on the plasma T level, but there was a significant reduction of plasma 11-KT level in 250 ng/L-exposed males at all the salinities ($p=0.004$) when compared to the control group (Figure 2.1D; Table 2.4).

2.4.1.5. Gonadal steroid production in males

There was a significant interaction of salinity and EE_2 when the testes were incubated in basal medium ($p<0.001$; data not shown). At 0 ppt, 50 ng/L of EE_2 produced a significant reduction in the basal production of gonadal T in males ($p=0.01$; Figure 2.3A) and there was a significantly greater T production in 250 ng/L-exposed males than in the 50 ng/L group in 25-OH cholesterol stimulated incubations ($p=0.002$; Figure 2.3A). At 16 ppt, there was a significant decrease in the production of T in 50 ($p<0.001$) and 250 ng/L ($p<0.001$) EE_2-exposed males compared to the controls when the gonads were incubated with 25-OH cholesterol (Figure 2.3B). At 32 ppt, there was a significant effect of EE_2 on the production of T in the testes ($p=0.01$); however, as a result of high variability, the Tukey's test could not discriminate which group(s) differ (Figure 2.3C; Table 2.4).

There was no interaction between salinity and EE_2 when production of 11-KT by pieces of testes was analyzed ($p=0.944$; data not shown), but there was a strong decrease in gonadal 11-KT caused by an increase of salinity ($p=0.016$; data not shown). There was no significant effect of EE_2 on 11-KT production at any salinity (0 ppt, $p=0.082$, Figure 2.3D; 16 ppt, $p=0.096$, Figure 2.3E; 32 ppt, $p=0.407$, Figure 2.3F; Table 2.4).

2.4.2. Effects of EE_2 under differing temperature conditions

2.4.2.1. Organ weights and somatic indices

There was no interaction between temperature and EE_2 ($p=0.399$), and no effect of temperature ($p=0.489$) or EE_2 ($p=0.522$) on female weight. In males, there was no interaction between the two main factors ($p=0.431$) and no effect of temperature ($p=0.943$) nor EE_2 (0.839) on body weight. There was no significant effect of temperature ($p=0.958$), nor effect of EE_2 ($p=0.341$), on female liver weight. Temperature significantly decreased male liver weight ($p=0.043$) and when males were kept at 26°C EE_2 significantly decreased liver weight ($p=0.013$). When female gonad weight was analyzed, there was no significant interaction between the two main factors ($p=0.122$), but there was a change in gonad weight with temperature increase ($p<0.001$). There was no interaction between the main factors ($p=0.068$), but temperature increment significantly increased male gonad weight with temperature, when males were kept at 18 ($p=0.029$) and 26°C ($p=0.001$), compared to 10°C (Table 2.2).

In females there was no interaction between temperature and EE_2 ($p=0.364$), but there was a significant decrease of LSI with the temperature increase ($p<0.001$). There was no effect of EE_2 on female LSI ($p=0.473$). There was no interaction between the two main factors when male LSI was analyzed ($p=0.614$). There was no significant effect of temperature on male LSI ($p=0.069$). EE_2 significantly affected male LSI ($p=0.024$), but as a result of high variability the post hoc test could not distinguish between exposure levels (Table 2.2).

There was no interaction between temperature and EE_2 ($p=0.072$) on female GSI, and no effect of EE_2 ($p=0.440$). Female GSI increased with temperature; at 26ºC female GSI was higher in the control group than at 18 or 10ºC ($p<0.001$). In males, there was significant interaction between the two main factors ($p=0.035$), and an increase in temperature significantly increased male GSI ($p=0.04$). EE_2 significantly decreased GSI ($p=0.01$) when males were exposed to 250 ng/L and were held at 26ºC (Table 2.2).

2.4.2.2. Plasma steroid analysis in females

There was neither an interaction between temperature and EE_2 ($p=0.694$; data not shown), nor an effect of temperature ($p=0.293$; Figure 2.4A), on female plasma T circulating levels. There was no effect of EE_2 ($p=0.463$) on female T plasma levels (Figure 2.4A). There was no interaction between temperature and EE_2 on plasma estradiol ($p=0.129$; data not shown). There was a strong effect of temperature on E_2 levels in plasma in the control group held at 18 and 26ºC compared to the control group at 10ºC ($p<0.001$; Figure 2.4B). At 18ºC and 26ºC there were 4- and 3-fold higher plasma E_2 levels, respectively, than E_2 levels in females kept at 10ºC (Figure 2.4B). There was a significant reduction in plasma E_2 when females were exposed to 250 ng/L of EE_2 at 18ºC ($p=0.015$; Figure 2.4B) (Table 2.5).

2.4.2.3. Gonadal steroid production in females

When analyzing the gonadal T production in ovaries, it was shown that there was a significant interaction between temperature and EE_2 ($p=0.036$; data not shown). At 10ºC, EE_2 caused a significant reduction of basal ($p<0.001$; Figure 2.5A), 25-OH cholesterol- ($p=0.001$; Figure 2.5A) and pregnenolone-stimulated ovarian T production ($p=0.004$; Figure 2.5A). At 18ºC, EE_2 did not affect production of T in the ovaries ($p=0.327$; Figure 2.5B). When females were kept at 26ºC, there was a significant reduction of female gonadal T when exposed to 250 ng/L of EE_2 but only when the gonads were incubated in the presence of 25-OH cholesterol ($p=0.004$; Figure 2.5C; Table 2.5).

There was no interaction between temperature and EE_2 for ovarian E_2 production ($p=0.537$; data not shown). There was a significant effect of temperature on the E_2 production ($p=0.002$; data not shown) in basal-incubated ovaries. Females maintained at 10ºC showed a reduction of E_2 production only in the presence of testosterone ($p=0.018$; Figure 2.5D). Mummichog females kept at 18ºC and exposed to 250 ng/L of EE_2 showed a significant reduction in ovarian E_2 production when the gonads were incubated with 25-OH cholesterol ($p=0.002$; Figure 2.5E); pregnenolone ($p<0.001$; Figure 2.5E) and testosterone ($p=0.006$; Figure 2.5E). At 26ºC, when pregnenolone and testosterone were present in the incubation media, there was a significant reduction in ovarian E_2 production in those fish exposed to 250 ng/L ($p=0.021$ and $p<0.001$, respectively; Figure 2.5F; Table 2.5).

2.4.2.4. Plasma steroid analysis in males

For male plasma T there was no interaction between temperature and EE_2 (p=0.606; data not shown) nor a significant effect of temperature on T levels (p=0.172). There was no significant effect of EE_2 on the plasma T (p=0.334; Figure 2.4C). There was no interaction between temperature and EE_2 (p=0.986; data not shown) nor effects of temperature on the plasma 11-KT levels (p=0.362). There was no effect of EE_2 on plasma 11-KT (p=0.109; Figure 2.4D; Table 2.6).

2.4.2.5. Gonadal steroid production in males

For male gonadal testosterone production, there was no interaction between temperature and EE_2 (p=0.052; data not shown) and no significant effect of temperature (p=0.170; data not shown). No significant effect of EE_2 on male testosterone production was found (p=0.107) when all the temperatures were combined (Figure 2.6A). There was no interaction between temperature and EE_2 for 11-KT production in males, (p=0.417; data not shown). However, there was a strong effect of temperature on 11-KT production in males ($p<0.001$, data not shown). At 10ºC EE_2 did not significantly affect 11-KT production (p=0.395; Figure 2.6B). At 18ºC there was a significant reduction in 11-KT production when fish were exposed to 250 ng/L of EE_2 and the testes were incubated in basal or 25-OH cholesterol media (p=0.015; Figure 2.6C). At 26ºC, there was no significant effect of EE_2 on 11-KT production in males (p=0.945; Figure 2.6D; Table 2.6).

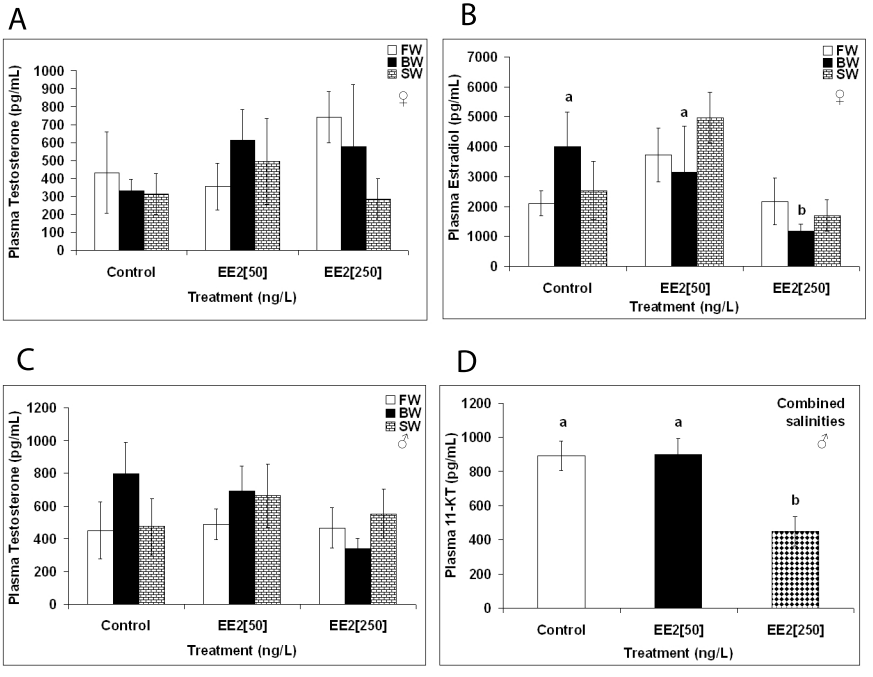
2.4.3 Effects of EE_2 on P450 aromatase A (cyp 19a) gene expression

There was no significant effect of EE_2 on P450 aromatase expression on those fish kept at 16 ppt and at 18ºC ($p=0.979$; Figure 2.7).

Table 2.2: Mean ± (SEM) weight, liver weight, liver somatic index (LSI) and gonadosomatic index (GSI) for adult female and male *Fundulus heteroclitus* exposed to EE_2 in differing salinities and differing temperature conditions. Differing lower case letters indicate significant differences for the EE_2 factor. Upper case letters indicate significant differences for the salinity or temperature factor.

	Salinity (ppt)	EE2 (ng/L)	N	Weight (g)	Liver Weight (g)	Gonad Weight (g)	LSI (%)	GSI (%)
Females	0	0	12	10.71±1.08	0.38±0.04	0.59±0.13	3.55±0.23	4.96±0.81
		50	12	9.27±0.70	0.42±0.05	0.59±0.10	4.45±0.34	6.32±1.09
		250	11	8.77±0.61	0.34±0.03	0.46±0.12	3.89±0.31	4.92±1.00
	16	0	12	9.24±0.85	0.30±0.03	0.54±0.12	3.35±0.22	5.56±0.99
		50	12	7.72±0.59	0.33±0.02	0.47±0.09	4.55±0.41	6.58±1.39
		250	12	8.60±0.65	0.29±0.02	0.40±0.08	3.43±0.20	4.36±0.7
	32	0	12	7.97± 0.51	0.35±0.03	0.39±0.08	4.45±1.08	5.08±1.09
		50	12	8.68±0.60	0.34±0.04	0.52±0.08	3.92±0.34	5.98±0.86
		250	12	9.24±0.52	0.37±0.02	0.45±0.09	4.16±0.27	4.64±0.82
Males	0	0	12	$5.82±0.37^{A}$	$0.22±0.02^{A}$	$0.07±0.01^{A}$	$3.83±0.34^{A,B}$	$1.20±0.17^{A}$
		50	12	$5.82±0.42^{A}$	$0.19±0.01^{A}$	$0.09±0.01^{A}$	$3.37±0.23^{A}$	$1.67±0.22^{A}$
		250	11	$4.86±0.39^{A}$	$0.16±0.01^{A}$	$0.05±0.01^{A}$	$3.47±0.33^{A}$	$1.07±0.12^{A}$
	16	0	12	$6.09±0.34^{A}$	$0.19±0.02^{A}$	$0.11±0.02^{A}$	$3.16±0.35^{A}$	$1.76±0.23^{A}$
		50	12	$6.47±0.71^{A}$	$0.19±0.02^{A}$	$0.13±0.03^{A}$	$3.03±0.22^{A}$	$1.89±0.25^{A}$
		250	12	$5.3±0.34^{A}$	$0.07±0.009^{A}$	$0.18±0.01^{A}$	$3.42±0.22^{A}$	$1.30±0.18^{A}$
	32	0	12	$8.68±0.60^{B}$	$0.35±0.03^{B}$	$0.40±0.09^{B}$	$4.45±0.18^{B}$	$5.08±1.09^{B}$
		50	12	$8.68±0.60^{B}$	$0.34±0.04^{B}$	$0.52±0.08^{B}$	$3.92±0.35^{B}$	$5.98±0.85^{B}$
		250	12	$9.24±0.51^{B}$	$0.37±0.20^{B}$	$0.45±0.09^{B}$	$4.16±0.27^{B}$	$4.64±0.82^{B}$
	Temp (°C)	**EE2 (ng/L)**	**N**	**Weight (g)**	**Liver Weight (g)**	**Gonad Weight (g)**	**LSI (%)**	**GSI (%)**
Females	10	0	12	8.41±0.64	$0.40±0.03^{A}$	$0.3±0.07^{A}$	$4.79±0.2^{A}$	$3.40±0.68^{A}$
		250	12	9.67±0.56	$0.42±0.22^{A}$	$0.68±0.21^{A}$	$4.43±0.23^{A}$	$6.21±1.62^{A}$
	18	0	12	9.24±0.85	$0.30±0.03^{A}$	$0.54±0.12^{A}$	$3.35±0.22^{B}$	$5.52±0.99^{A}$
		250	12	8.62±0.65	$0.28±0.02^{B}$	$0.4±0.08^{A}$	$3.44±0.2^{A}$	$4.37±0.7^{A}$
	26	0	12	9.15±0.82	$0.30±0.02^{A}$	$1.13±0.17^{B}$	$3.43±0.22^{B}$	$11.95±1.05^{B}$
		250	12	9.64±0.65	$0.40±0.08^{A}$	$0.91±0.16^{A}$	$4.25±0.9^{A}$	$9.02±1.59^{A}$
Males	10	0	12	5.72±0.46	$0.21±0.02^{A}$	$0.06±0.01^{A,a}$	3.80±0.23	$1.11±0.12^{A,a}$
		250	12	5.86±0.38	$0.26±0.02^{A}$	$0.06±0.01^{A,a}$	4.57±0.30	$1.05±0.12^{A,a}$
	18	0	12	6.09±0.34	$0.19±0.02^{A,B}$	$0.11±0.02^{B,a}$	3.16±0.35	$1.76±0.24^{A,B,a}$
		250	12	5.31±0.34	$0.18±0.01^{B}$	$0.07±0.01^{A,b}$	3.42±0.22	$1.30±0.18^{A,a}$
	26	0	12	5.43±0.56	$0.13±0.01^{B}$	$0.13±0.01^{B,a}$	2.56±0.23	$2.20±0.21^{B,a}$
		250	11	5.82±0.70	$0.22±0.07^{A,B}$	$0.07±0.01^{A,b}$	3.80±1.04	$1.22±0.22^{A,b}$

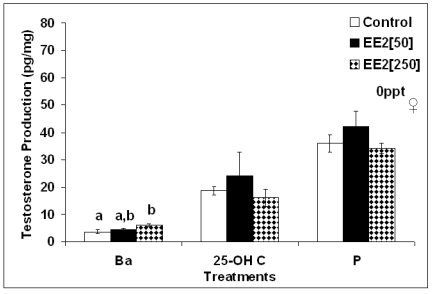
Figure 2.1: Effects of EE_2 (50 and 250 ng/L) on plasma steroid levels in mummichog (*Fundulus heteroclitus*) kept in differing saline conditions (0, 16 and 32 ppt). Bars represent means $\pm$ SEM. Differing letters indicate significant differences. N = 4 tanks per treatment. A: Effects of EE_2 on female plasma testosterone ($p=0.837$). B: Effects of EE_2 on female plasma estradiol ($p=0.045$). C: Effects of EE_2 on male plasma testosterone ($p=0.584$). D: Effects of EE_2 on male plasma 11-ketotestosterone (11-KT) with salinity data combined ($p=0.004$). FW = fresh water (0 ppt); BW = brackish water (16 ppt); SW = salt water (32 ppt). LOG = data log transformed prior to statistical analysis.


A
Plasma Testosterone (pg/mL)
1000
900
800
700
600
500
400
300
200
100
0
FW
BW
SW
♀
Control
EE2[50]
EE2[250]
Treatment (ng/L)
B
Plasma Estradiol (pg/mL)
7000
6000
5000
4000
3000
2000
1000
0
a
a
b
FW
BW
SW
♀
Control
EE2[50]
EE2[250]
Treatment (ng/L)
C
Plasma Testosterone (pg/mL)
1200
1000
800
600
400
200
0
FW
BW
SW
♂
Control
EE2[50]
EE2[250]
Treatment (ng/L)
D
Plasma 11-KT (pg/mL)
1200
1000
800
600
400
200
0
a
a
b
Combined salinities
♂
Control
EE2[50]
EE2[250]
Treatment (ng/L)

LOG

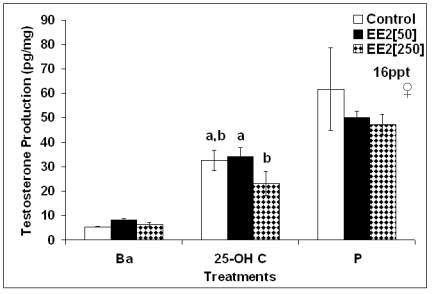
Figure 2.2: Effects of EE_2 (50 and 250 ng/L) on steroid production in ovarian pieces incubated from mummichog (*Fundulus heteroclitus*) held in differing saline conditions (0, 16 and 32 ppt). Bars represent means $\pm$ SEM. Differing letters indicate significant differences. N = 4 tanks per treatment. Ba = basal medium; 25-OH C = 25-OH cholesterol-stimulated medium; P = pregnenolone-stimulated medium; and T = testosterone-stimulated medium. A: Effects of EE_2 on ovarian testosterone production in females kept at 0 ppt ($p=0.036$). B: Effects of EE_2 on ovarian testosterone production in females kept at 16 ppt ($p=0.649$). C: Effects of EE_2 on ovarian testosterone production in females kept at 32 ppt ($p<0.001$ for 50ng/L of EE_2; $p=0.005$ for 250ng/L of EE_2). D: Effects of EE_2 on ovarian estradiol production with salinity data combined ($p<0.001$ for Ba; $p=0.004$ for 25-OH C; $p<0.001$ for P and $p=0.029$ for T). FW = fresh water (0 ppt); BW = brackish water (16 ppt); SW = salt water (32 ppt).

A



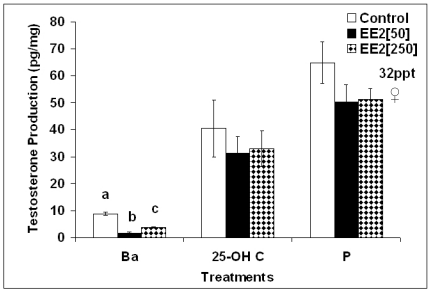
NP

B



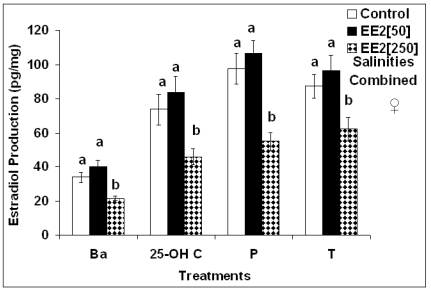
NP

C



LOG

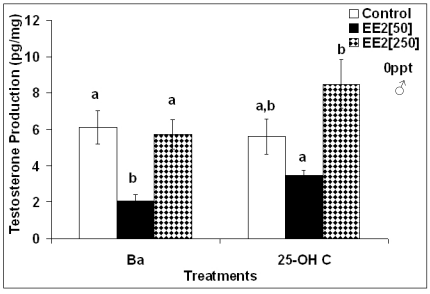
D



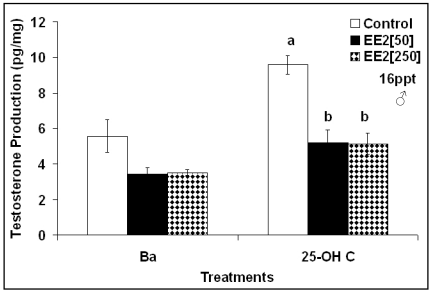
LOG

Figure 2.3: Effects of EE_2 (50 and 250 ng/L) on steroid production in pieces of testes incubated from mummichog (*Fundulus heteroclitus*) held in differing saline conditions (0, 16 and 32 ppt). Bars represent means $\pm$ SEM. Differing letters indicate significant differences. N = 4 tanks per treatment. Ba = basal medium; 25-OH C = 25-OH cholesterol-stimulated medium. A: Effects of EE_2 on testosterone production in males kept at 0 ppt ($p=0.01$). B: Effects of EE_2 on testosterone production in males kept at 16 ppt ($p<0.001$ when exposed to 50 and 250 ng/L of EE_2). C: Effects of EE_2 on testosterone production in males kept at 32 ppt ($p=0.01$). D: Effects of EE_2 on 11-ketotestosterone (11-KT) production in fish kept at 0 ppt ($p=0.082$). E: Effects of EE_2 on 11-KT production in males kept at 16 ppt ($p=0.096$). F: Effects of EE_2 on 11-KT production in males kept at 32 ppt ($p=0.407$). LOG = data log transformed prior to statistical analysis.

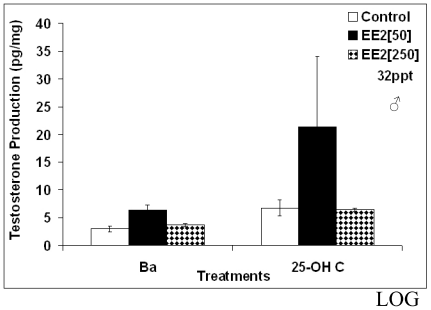
A


Control
EE2[50]
EE2[250]
0ppt
♂
Testosterone Production (pg/mg)
0
2
4
6
8
10
12
a
b
a
a,b
a
b
Ba
25-OH C
Treatments

B

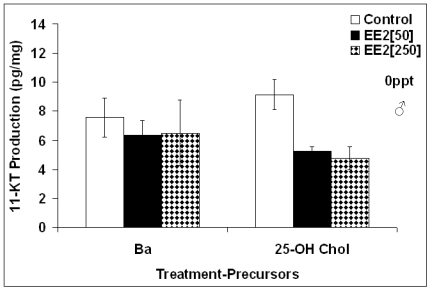

Control
EE2[50]
EE2[250]
16ppt
♂
Testosterone Production (pg/mg)
0
2
4
6
8
10
12
a
b
b
Ba
25-OH C
Treatments

C

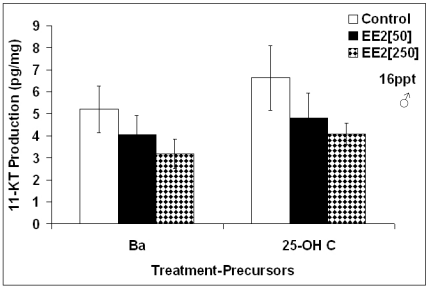

Control
EE2[50]
EE2[250]
32ppt
♂
Testosterone Production (pg/mg)
0
5
10
15
20
25
30
35
40
Ba
25-OH C
Treatments

LOG

D


Control
EE2[50]
EE2[250]
0ppt
♂
11-KT Production (pg/mg)
0
2
4
6
8
10
12
14
Ba
25-OH Chol
Treatment-Precursors

E


Control
EE2[50]
EE2[250]
16ppt
♂
11-KT Production (pg/mg)
0
1
2
3
4
5
6
7
8
9
Ba
25-OH C
Treatment-Precursors

F

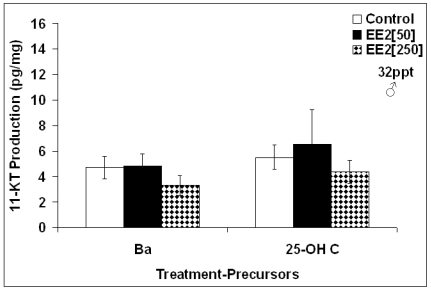
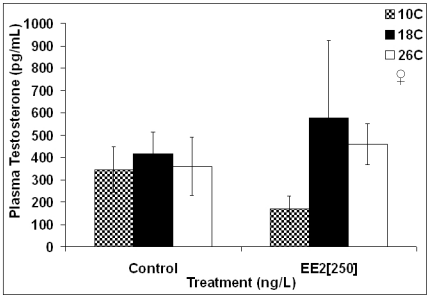
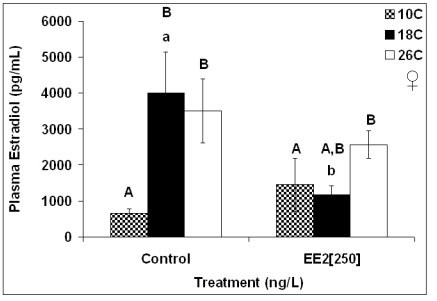

Control
EE2[50]
EE2[250]
32ppt
♂
11-KT Production (pg/mg)
0
2
4
6
8
10
12
14
16
Ba
25-OH C
Treatment-Precursors

Figure 2.4: Effects of 250 ng/L of EE_2 on plasma steroid levels in mummichog (*Fundulus heteroclitus*) kept in differing temperature conditions (10, 18 and 26ºC). Bars represent means $\pm$ SEM. Differing lower case letters indicate significant differences for the EE_2 factor. N = 4 tanks per treatment. Upper case letters indicate the effects of temperature on plasma steroid levels. A: Effects of EE_2 on female plasma testosterone ($p=0.463$). B: Effects of EE_2 on female plasma estradiol ($p=0.015$). C: Effects of EE_2 on male plasma testosterone ($p=0.334$). D: Effects of EE_2 on male plasma 11-ketotestosterone ($p=0.109$). LOG = data log transformed prior to statistical analysis.

A

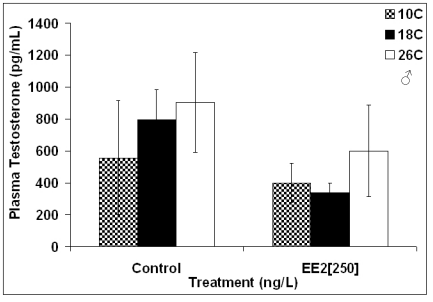

Plasma Testosterone (pg/mL)
1000
900
800
700
600
500
400
300
200
100
0
10C
18C
26C
♀
Control
EE2[250]
Treatment (ng/L)

B

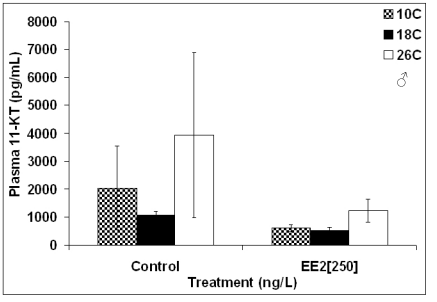

Plasma Estradiol (pg/mL)
6000
5000
4000
3000
2000
1000
0
B
a
B
A
A
A,B
b
B
10C
18C
26C
♀
Control
EE2[250]
Treatment (ng/L)

LOG

C


Plasma Testosterone (pg/mL)
1400
1200
1000
800
600
400
200
0
10C
18C
26C
♂
Control
EE2[250]
Treatment (ng/L)

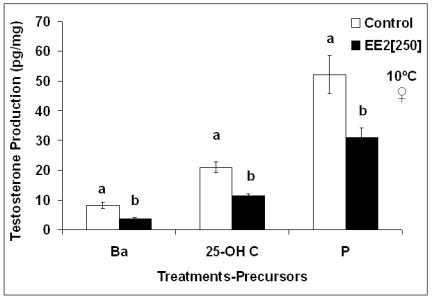
D


Plasma 11-KT (pg/mL)
8000
7000
6000
5000
4000
3000
2000
1000
0
10C
18C
26C
♂
Control
EE2[250]
Treatment (ng/L)

LOG

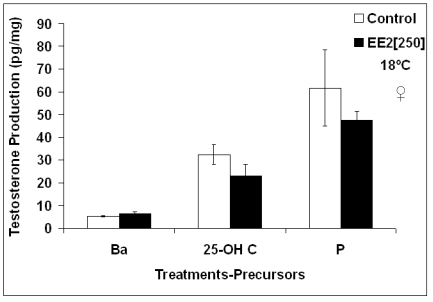
Figure 2.5: Effects of 250 ng/L of EE_2 on steroid production in ovarian pieces incubated from mummichog (*Fundulus heteroclitus*) held in differing temperature conditions (10, 18 and 26ºC). Bars represent means $\pm$ SEM. Differing letters indicate significant differences. N = 4 tanks per treatment. Ba = basal medium; 25-OH C = 25-OH cholesterol-stimulated medium; P = pregnenolone-stimulated medium; and T = testosterone-stimulated medium. A: Effects of EE_2 on ovarian testosterone production in females kept at 10ºC ($p<0.001$). B: Effects of EE_2 on ovarian testosterone production in females kept at 18ºC ($p=0.327$). C: Effects of EE_2 on ovarian testosterone production in females kept at 26ºC ($p=0.004$). D: Effects of EE_2 on ovarian estradiol production in females kept at 10ºC ($p=0.018$). E: Effects of EE_2 on ovarian estradiol production in females kept at 18ºC ($p=0.002$ for 25-OH C; $p<0.001$ for P and $p=0.006$ for T). F: Effects of EE_2 on ovarian estradiol production in females kept at 26ºC ($p=0.021$ for P and $p<0.001$ for T). LOG = data log transformed prior to statistical analysis.

A



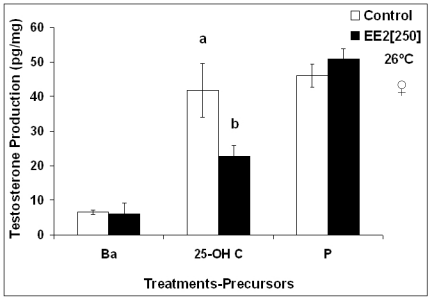
LOG

B

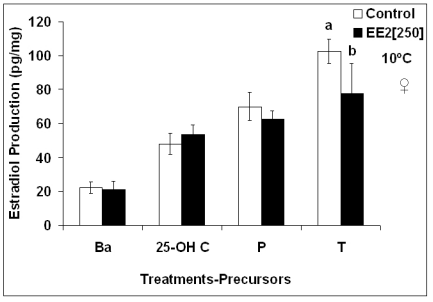


LOG

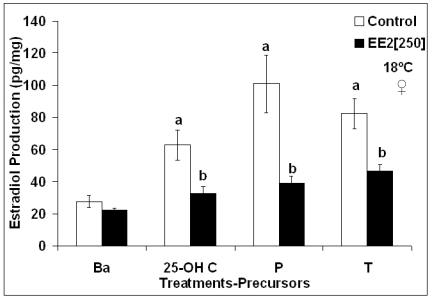
C



D

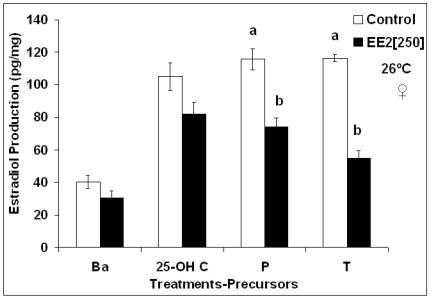


E



LOG

F



LOG

Figure 2.6: Effects of 250 ng/L of EE_2 on steroid production in pieces of testes incubated from mummichog (*Fundulus heteroclitus*) held in differing temperature conditions (10, 18 and 26ºC). Bars represent means $\pm$ SEM. Differing letters indicate significant differences. N = 4 tanks per treatment. Ba = basal medium; 25-OH C = 25-OH cholesterol-stimulated medium. A: Effects of EE_2 on testosterone production in males ($p=0.107$). B: Effects of EE_2 on 11-ketotestosterone production in males kept at 10ºC ($p=0.395$). C: Effects of EE_2 on 11-ketotestosterone production in males kept at 18ºC ($p=0.015$). D: Effects of EE_2 on 11-ketotestosterone production in fish kept at 26ºC ($p=0.945$). LOG = data log transformed prior to statistical analysis.

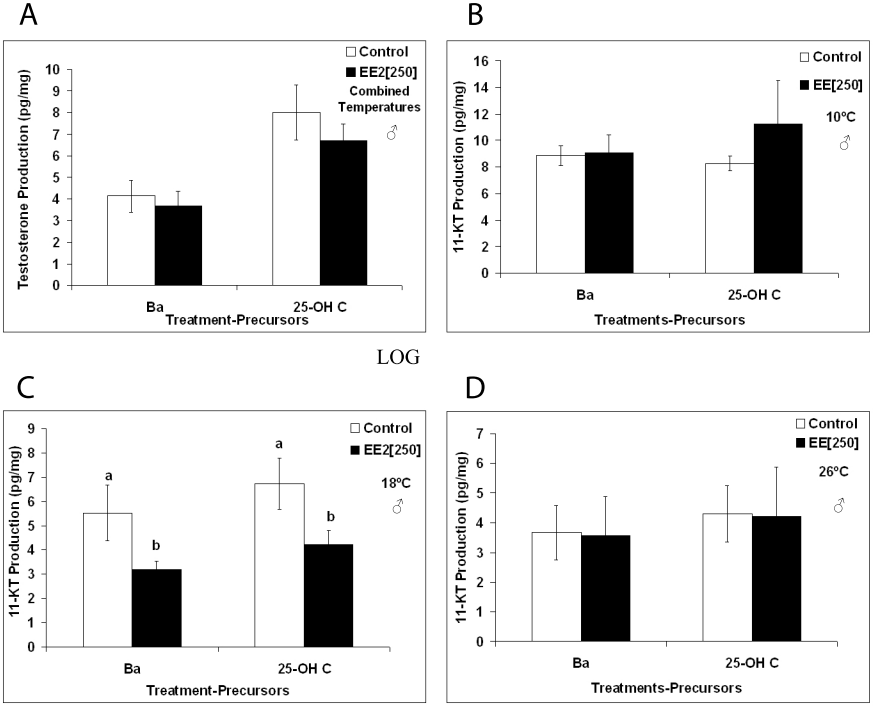

A
Control
EE2[250]
Combined Temperatures
♂
Testosterone Production (pg/mg)
10
9
8
7
6
5
4
3
2
1
0
Ba
25-OH C
Treatment-Precursors
B
Control
EE[250]
10°C
♂
11-KT Production (pg/mg)
16
14
12
10
8
6
4
2
0
Ba
25-OH C
Treatments-Precursors
LOG
C
Control
EE2[250]
18°C
♂
11-KT Production (pg/mg)
9
8
7
6
5
4
3
2
1
0
a
b
a
b
Ba
25-OH C
Treatment-Precursors
D
Control
EE[250]
26°C
♂
11-KT Production (pg/mg)
7
6
5
4
3
2
1
0
Ba
25-OH C
Treatments-Precursors

Table 2.3: Summary of effects of EE_2 (50 and 250 ng/L) on plasma steroids and gonadal biosynthesis of steroids in female mummichog *(Fundulus heteroclitus)* held in differing saline conditions (0, 16 and 32 ppt). Ba = basal; 25C = 25-OH cholesterol-stimulated medium; P = pregnenolone-stimulated medium and T = testosterone-stimulated medium; Sa = salinity. ↑ indicates significant increase; ↓ indicates significant decrease; = indicates no difference.

Steroid																
		Testosterone						17β-Estradiol								
	EE_2 (ng/L) / Sa (ppt)	50			250			50				250				
Plasma Steroids	0	=			=			=				=				
	16	=			=			=				↓				
	32	=			=			=				=				
Gonadal Steroid Production	Precursor / Sa (ppt)	Ba	25 C	P	Ba	25 C	P	Ba	25 C	P	T	Ba	25 C	P	T	
	0	=	=	=	↑	=	=	=	=	=	=	↓	↓	↓	↓	
	16	=	=	=	=	↓	=	=	=	=	=	↓	↓	↓	↓	
	32	↓	=	=	↓	=	=	=	=	=	=	↓	↓	↓	↓	

Table 2.4: Summary of effects of EE_2 (50 and 250 ng/L) on plasma steroids and gonadal biosynthesis of steroids in male mummichog *(Fundulus heteroclitus)* held in differing saline conditions (0, 16 and 32 ppt). Ba = basal; 25C = 25-OH cholesterol-stimulated medium; Sa = salinity. ↑ indicates significant increase; ↓ indicates significant decrease; = indicates no difference.

Steroids									
		Testosterone				11-Ketotestosterone			
Plasma Steroids	EE_2 (ng/L) / Sa (ppt)	50		250		50		250	
	0	=		=		=		↓	
	16	=		=		=		↓	
	32	=		=		=		↓	
Gonadal Steroid Production	Precursor / Sa (ppt)	Ba	25C	Ba	25C	Ba	25C	Ba	25C
	0	↓	=	=	↑	=	=	=	=
	16	=	=	↓	↓	=	=	=	=
	32	=	=	=	=	=	=	=	=

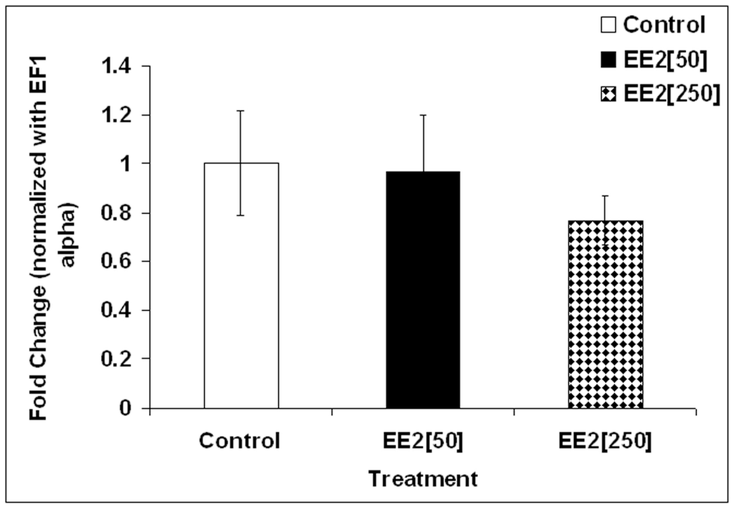
Table 2.5: Summary of effects of EE_2 (250 ng/L) on plasma steroids and gonadal biosynthesis of steroids in female mummichog *(Fundulus heteroclitus)* held in differing temperature conditions (10, 18 and 26ºC). Ba = basal; 25C = 25-OH cholesterol-stimulated medium; P = pregnenolone-stimulated medium and T = testosterone-stimulated medium. ↓ indicates significant decrease; = indicates no difference.

		Steroids						
		Testosterone			17β-Estradiol			
Plasma Steroids	EE_2 (ng/L) / Temp (ºC)	250			250			
	10	=			=			
	18	=			↓			
	26	=			=			
Gonadal Steroid Production	Precursor / Temp (ºC)	Ba	25C	P	Ba	25C	P	T
	10	↓	↓	↓	=	=	=	↓
	18	=	=	=	=	↓	↓	↓
	26	=	↓	=	=	=	↓	↓

Table 2.6: Summary of effects of EE_2 (250 ng/L) on plasma steroids and gonadal biosynthesis of steroids in male mummichog *(Fundulus heteroclitus)* held in differing temperature conditions (10, 18 and 26ºC). Ba = basal; 25C = 25-OH cholesterol-stimulated medium; P = pregnenolone-stimulated medium and T = testosterone-stimulated medium. ↓ indicates significant decrease; = indicates no difference.

		Steroids			
		Testosterone		11-Ketotestosterone	
Plasma Steroids	EE_2 (ng/L) / Temp (ºC)	250		250	
	10	=		=	
	18	=		=	
	26	=		=	
Gonadal Steroid Production	Precursor / Temp (ºC)	Ba	25C	Ba	25C
	10	=	=	=	=
	18	=	=	↓	↓
	26	=	=	=	=

Figure 2.7: Effects of EE_2 on ovarian aromatase gene expression of mummichog (*Fundulus heteroclitus*) at 16ppt and 18ºC. Bars represent means $\pm$ SEM. ($p=0.979$)


Control
EE2[50]
EE2[250]
Fold Change (normalized with EF1 alpha)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
EE2[50]
EE2[250]
Treatment

2.5. Discussion

This study advances our understanding of effects of EE_2 and environmental parameters on reproductive endocrine status in fish. While salinity alone had limited impact, effects of EE_2 at 250 ng/L confirmed that EE_2 depresses reproductive endocrine endpoints in fish (MacLatchy *et al.*, 2003; Martyniuk *et al*., 2006; Hogan *et al.*, 2010). These depressions occurred across all salinities as indicated by decreased plasma E_2 and E_2 production in females and decreased plasma 11-KT in males. Temperature was further established as a significant environmental factor regulating northern mummichog (*Fundulus heteroclitus macrolepidotus*) reproduction (McMullin *et al.*, 2009). Increased temperature triggered gonadal growth and maturation in both females and males. In addition, plasma E_2 levels and E_2 production in females increased with temperature, whereas 11-KT production in males decreased with temperature. Most notably, EE_2 counteracted temperature-related effects, depressing the temperature-induced increases in gonad size in males. The lack of effects of salinity in general could be protective in a species that reproduces in a highly-variable saline environment. The responses to EE_2 at higher temperatures could be due to temperature-related increases in uptake of EE_2 (Blewett, 2011) and/or increased susceptibility during temperature-induced gonadal maturation.

2.5.1. Exposure concentrations

The EE_2 concentrations used in our work were chosen for two reasons. Firstly, 50 ng/L of EE_2 in a static exposure is close to the environmentally-relevant concentrations found in Canadian and American aquatic environments (EE_2 concentrations as high as 42 ng/L and averaging 0.2 and 7 ng/L EE_2; Desbrow *et al*., 1998; Ternes *et al*., 1999). As EE_2 is very hydrophobic (Log K_{ow}= 4.15; Teske & Arnold, 2008) and tends to get attached to the airline tubing and the glass walls of the tank, we assumed the 50 ng/L nominal concentration would decrease to a final concentration of EE_2 in the exposure tanks close to environmental relevance. This has been shown in earlier studies, in which EE_2 final concentrations were 58% to 84% of the original concentration of 0.2, 1, 4, 16 and 64 ng/L (Länge *et al.*, 2001); 10-20% of the original nominal 100 ng/L (Peters *et al.*, 2007); 60% of 20 ng/L (Björkblom *et al*., 2009); and 70% and 50% of 100 and 500 ng/L, respectively (Hogan *et al.* 2010). Secondly, 250 ng/L of EE_2 has been demonstrated to provide mechanistic information on the effects of EE_2 on steroidogenesis in mummichog (Hogan *et al.*, 2010). While water samples were taken to quantify actual EE_2 exposure levels in the present study, issues with the quality assurance/quality control in the analyzing laboratory meant no values are available for reporting purposes.

Previous studies have indicated that higher concentrations of EE_2 are needed to produce endocrine effects in the estuarine sheepshead minnow (*Cyprinodon variegatus Lacépède*; Zillioux *et al.*, 2001) and mummichog (MacLatchy *et al.*, 2003; Peters *et al*., 2007, Hogan *et al*., 2010) than in freshwater species. In mummichog, the requirement for higher EE_2 exposure concentrations to cause reproductive endocrine effects could be due

to one or more factors, including differences in EE_2 availability, uptake, tissue distribution, metabolism or receptor affinity. Recent studies have indicated that EE_2 availability is the same in 0 or 16 ppt salinity (Nadon, 2010). Gill uptake of EE_2 is greater in mummichog at 16 ppt rather than 0 or 32 ppt; this has been hypothesized to be due to the expanded diffusion distance for EE_2 across mummichog gills in brackish water (Blewett, 2011). At 16 ppt the diffusion distance is small due to the presence of apical pores on the outer lamellae, whereas at 0 ppt there is a predominance of thick cuboidal cells mixed with pavement cells and inactive seawater chlorine cells (SWCC) (Marshall *et al*., 1997; Katoh *et al*., 2001; Laurent *et al*., 2006). At 32 ppt there is an increased number of SWCC to cope with the ionic balance. An increase in gill thickness in both fresh and saltwater conditions slows the diffusion rate (Philpott & Copeland, 1963; Laurent *et al.*, 2006). As EE_2 uptake rates correspond with O_2 uptake (Blewett, 2011), the rates of uptake being greater at 16 ppt than 0 ppt and 32 ppt due to gill structure is further supported.

In regard to tissue distribution and metabolism, EE_2 is distributed very quickly to the liver, gall bladder and gut in mummichog following uptake (Blewett, 2011). The distribution pattern indicates a high rate of metabolism and potentially low effective concentrations in target tissues. This distribution pattern differs between mummichog and rainbow trout held at 0 and 16 ppt (Blewett, 2011), demonstrating species differences in EE_2 tissue distribution and elimination rates. Mummichog are known to have high tolerance for various contaminants (Burnett *et al*., 2007; Lister *et al*., 2011) and high rates of metabolism and elimination could account for some of this tolerance and low comparative sensitivity to EE_2.

EE_2 has high affinity for the estrogen receptor (Segner *et al.*, 2003) and stimulates ER-mediated effects, such as vitellogenin gene expression (Björkblom, *et al.*, 2009) and protein production (Osborne *et al*., 2007). ER gene structure varies among fish species. Phylogenetic analysis of ER gene structure by Orlando *et al.* (2006) showed that ERα and ERβ of mummichog is closer to that of Japanese medaka and mangrove killifish (*Kryptolebias marmoratus*) than to goldfish or rainbow trout. One could then hypothesize that Japanese medaka will also need a higher concentration of EE_2 to produce an endocrine response. Indeed, previous studies have demonstrated that higher concentrations of EE_2 (50-488 ng/L) are needed to affect the reproductive endocrinology of Japanese medaka (Seki *et al,* 2002; Ma *et al.,* 2005; Kashiwada *et al*., 2007). Therefore, it would be interesting for future experiments to analyze the effects of EE_2 on species close and distant phylogenetically to mummichog, focusing in the interaction between EE_2 and the ER, to determine whether ER gene structure, and ultimately ER structure, has roles in determining the effective concentration of EE_2 required to stimulate reproductive endocrine responses in fish.

2.5.2. Salinity exposure

According to our results, salinity is not a major factor affecting mummichog reproductive endocrine status since there are not marked correlations between salinity and somatic indices, plasma steroids or steroid production. Higher salinity levels increased male body weight, liver weight, gonad weight, LSI and GSI. The increases in male mummichog somatic indices when salinity was raised could be related to increased

food intake or improved energy conversion for osmoregulation (Tseng & Hwang, 2008). However, similar changes were not observed in female somatic endpoints, perhaps due to the amount of energy diverted at any salinity to reproduction. Food was not limiting in the exposures, therefore eliminating food availability as a factor in the results.

Overall, salinity did not affect the endocrine response of mummichog to EE_2, as 250 ng/L of EE_2 in the three salinities tested decreased female E_2 production and male circulating 11-KT levels equally at all salinities. There was a significant interaction between salinity and EE_2 when we analyzed female gonadal T and male gonadal T production. Comparison of female T production between 0 ppt and 16 ppt, and 0 ppt and 32 ppt, showed 1.64- and 2-fold increases, respectively, with increasing salinity, but no change in male T production. Additionally, a salinity variation between 0 ppt and 32 ppt caused a 1.6-fold depression in 11-KT production in males. Nadon (2010) has recently demonstrated greater induction of vitellogenin 1 and 2 gene expression in mummichog at 16 ppt compared to 0 ppt of salinity. Coupled with increased uptake of EE_2 at 16 ppt of salinity compared to 0 ppt and 32 ppt (Blewett, 2011), increased effects of EE_2 at 16 ppt might have been expected. A greater endocrine response at 16 ppt was detected for plasma E_2 in females, as levels at 16 ppt were significantly decreased compared to levels at 0 and 32 ppt. However, this effect was minor when all the results are considered. The limited responses in reproductive endocrine endpoints at different salinities, however, reinforces the hypothesis that tissue distribution in mummichog to the liver, gall bladder and gut at all salinities, as demonstrated by Blewett (2011), diminishes non-hepatic tissue distribution (including gonads) even if uptake is higher at particular salinities.

2.5.3. Temperature exposure

In the present study, we found that temperature is a major environmental factor regulating mummichog reproductive physiology at different levels of biological organization. An increase in temperature triggered gonadal growth and maturation in both females and males. GSI increased in both females and males with increases in temperature, perhaps initiating a spawning cycle, and confirming the importance of temperature as a factor controlling reproduction in northern mummichog. Our results support the findings of McMullin *et al.* (2009), who also found in a field study that the reproductive cycle of northern mummichog is chiefly regulated by temperature and that females are more responsive to temperature than males during the spawning season. Increasing temperature decreased LSI in mummichog females and decreased liver weight in males. The variation in LSI and liver weight is possibly due to the use of stored energy in the liver. Several fish species use stored liver fat as an energy source during spawning periods (Kott, 1971; Overton & Van der Avyle, 2005; McMullin *et al.*, 2009).

Plasma E_2 levels and E_2 production in females increased with temperature, whereas male 11-KT production decreased with temperature. Females in 0 ng/L of EE_2 kept at 18 and 26ºC had 4- and 3-fold higher plasma E_2 levels than females at 10ºC. There was a significant depression on the production of E_2 at 10ºC only when testosterone was present in the incubation media. Our results indicate that females are more responsive to a temperature variation than males, since temperature regulated their reproductive status at several levels, whereas in males temperature modified the production of 11-KT but not the plasma levels.

EE_2 at 250 ng/L significantly increased male liver weight in fish held at 26°C. Estrogen exposure has previously been shown to increase liver weight and gonad weight in fish (Andersson *et al*., 2007). Liver weight increases are presumably due to increased metabolic activity required by the liver tissue to clear the estrogen and/or produce large quantities of the ER-mediated hepatic lipoprotein vitellogenin. Male GSI were depressed when exposed to 250 ng/L of EE_2 and held at 26°C. Male mummichog GSI was also significantly decreased by exposure to 250 ng/L of EE_2 when held at 18°C for 14 days (Hogan *et al.*, 2010) confirming studies with other fish species (e.g., three-spined stickleback, *Gasterosteus aculeatus*; Andersson *et al*., 2007).

In our temperature exposure, plasma E_2 was depressed when fish held at 18ºC were exposed to 250 ng/L of EE_2. Effects of EE_2 have been shown to depend on the levels of circulating E_2 (Kime *et al.*, 1999); because plasma E_2 was lower at 10ºC than at 18 or 26ºC, this could alter EE_2's effects. EE_2 depressed the production of E_2 when the ovaries were incubated in 25-OH cholesterol-, pregnenolone-, and testosterone-stimulated media at 18ºC, and when the ovaries were incubated in pregnenolone- and testosterone-stimulated medium at 26ºC. These results corroborate what we found in females in the salinity exposure experiments; that is, that EE_2's effects are downstream of cholesterol mobilization, and downstream of T to E_2 conversion. Increased uptake of EE_2 at higher temperatures (Blewett, 2011) could account for increased effects on steroidogenesis at higher temperatures.

2.5.4. Interpretation of effects of EE_2 on steroidogenesis and plasma steroids levels

In the present salinity and temperature exposures, the effect of EE_2 on female and male gonadal T production was highly variable. However, 250 ng/L of EE_2 markedly depressed E_2 production in females and 11-KT in males. Previous studies conducted in different species support our findings and corroborate EE_2's disruptive effects on fish reproductive endocrine status by affecting terminal steroid production. When Condeça & Canario (1999) exposed sea bream to 15 mg of EE_2/kg of diet for 37 and 112 d, they found that there was a significant increase in the production of both T and E_2. Juvenile turbot exposed to 3.5 and 75 ng/L of EE_2 for 15 d had a significant decrease in the production of 11-KT in males (Labadie & Budzinski, 2006). Previous studies with mummichog have shown that 500 ng/L of EE_2 reduced female T and E_2 production and male T and 11-KT production (MacLatchy *et al*., 2003). As well, 100 ng/L of EE_2 depressed T and E_2 production in females (Peters *et al.*, 2007). These latter two works indicate that terminal steroid biosynthesis in mummichog is affected by EE_2.

Similar to many other studies in mummichog (MacLatchy *et al*., 2003; Sharpe *et al.*, 2004; Peters *et al*., 2007; Hogan *et al*., 2010), plasma T and gonadal T production were not parallel in females and males, and neither were plasma and gonadal 11-KT in males. At 0 and 16 ppt the depressions caused by EE_2 on male 11-KT production were altered but at $p<0.1$ rather than $p<0.05$ ($p_{0ppt} = 0.082$; $p_{16ppt} = 0.096$). A parallelism between plasma steroids and gonadal steroid production indicates a potential mechanistic linkage between effects of EE_2 on gonadal steroidogenesis and circulating steroid levels.

An alteration in plasma steroids indicates a true endocrine effect, whereas if the disruptive effect is only on the gonadal production, the overall endocrine status of the animal may not be affected (Thomas, 1988; Munkittrick *et al.*, 1991; Munkittrick *et al.*, 1992; Hecker *et al*., 2002; Coe *et al*. 2008). The differences observed between the plasma and the gonadal T and 11-KT may be due to homeostatic mechanisms that regulate the T and 11-KT levels in the plasma. It has been demonstrated that the adrenal and the interrenal gland under the influence of gonadotrophins are able to produce T and 11-KT to compensate for a reduction in production by the gonads (Idler & McNab, 1967; Vermeulen *et al.*, 1994; Gazolla & Borella, 1997). As well, alterations in clearance rates of plasma steroids can be altered to maintain plasma steroid in homeostatic ranges (MacLatchy *et al*., 1997). The effect of EE_2 on female plasma E_2 and gonadal production demonstrates a synchronization of effects at the plasma and gonadal level. The parallelism observed for female E_2 was previously reported in mummichog by MacLatchy *et al.* (2003) and Peters *et al.* (2007).

2.5.5. Steroidogenic precursors as a useful experimental tool

Using steroidogenic precursors in the *in vitro* incubations helped us to determine that the effect of EE_2 in females is at the T to E_2 and in males at the T to 11-KT conversion steps of steroidogenesis. As previously demonstrated, the use of intermediates is a useful tool to determine the mechanistic effects of EE_2 or other contaminants (McMaster *et al.*, 1995; Hogan *et al.*, 2010). We have definitively demonstrated that in female mummichog, EE_2 alters steroidogenesis as far downstream as aromatase. This

supports and furthers the previous work of Hogan *et al.* (2010), in which exposure of EE_2 (250 ng/L nominal concentration) resulted in depressed gonadal T downstream of cholesterol mobilization. Sea bream gonads incubated with androstenedione, after *in vivo* exposure to 15 mg of EE_2/kg of diet, increased significantly the production of E_2 and T, demonstrating that the effect of EE_2 in sea bream was downstream of androstenedione to T conversion (Condeça & Canario, 1999). The value of this experimental design is that it pinpoints where further efforts to determine modes of action of EE_2 on steroidogenesis should be focused; for example, whether the effects are mediated by genomic or non genomic effects (Garcia-Reyero *et al.,* 2009). In mummichog, a limited but growing range of molecular tools exists, such as primer sequences for gonadal steroidogenic enzymes (Burnett *et al*., 2007; Lister *et al*., 2011).

2.5.6. Ovarian P450 aromatase A (cyp 19a) gene expression

EE_2 interacts with the ER (Condeça & Canario, 1999; Segner *et al.*, 2003), triggering genomic (Filby *et al.*, 2007) and non-genomic responses (Legler *et al.*, 2002; Thomas & Doughty, 2004; Vasudevan & Pfaff, 2008) in fish. In our study, EE_2 depressed ovarian T to E_2 production without altering aromatase A gene expression. A possible explanation could be that the effect of EE_2 is post-transcriptional. These potential mechanisms include regulation of mRNA half-life, control of translation, protein half-life, regulation of protein activity and post-translational modifications (Denslow *et al*., 2001), none of which were analyzed in this study. In the future, more research should be

done to investigate these potential mechanisms and to develop the tools necessary to do so.

2.5.7. Conclusions

In conclusion, our work is the first to demonstrate, by using steroidogenic precursors, that the effect of EE_2 in mummichog is most pronounced on final conversion of T to the terminal steroids (E_2 and 11-KT) in female and male mummichog. We were also able to demonstrate that temperature plays an important role in mummichog reproductive endocrine regulation, confirming the role of temperature in northern mummichog reproduction (MacLatchy *et al.*, 2005; McMullin *et al.*, 2009). EE_2 was demonstrated to be a potent endocrine disruptor since it is a strong antagonist to the effects of temperature. In estuaries, initiation of reproductive development in northern mummichog is linked with increases in water temperature (McMullin *et al.*, 2009). Reproductive endpoints assessed in this study appear to be generally robust when challenged by different salinities but not EE_2. There was minor evidence that reproductive endocrine endpoints are affected to a greater degree by EE_2 exposure at 16 ppt as previously demonstrated for direct receptor-mediated responses (vitellogenin 1 and 2 gene expression; Nadon, 2010). Overall, EE_2 retards reproductive development and reproductive endocrine status with the potential for population-level effects in wild fish, including during sensitive periods of gonadal recrudescence. Our study also indicates that experimental designs of standardized tests for screening EDSs need to be carefully chosen to ensure that interactions between environmental factors and the EDSs are being considered.

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Chapter 3

General Discussion and Future Directions

3.1. Relevant findings

This work corroborates the findings of McMullin *et al*. (2009) that temperature regulates northern mummichog (*Fundulus heteroclitus macrolepidotus*) reproductive status. Increasing temperature stimulates gonadal growth and steroidogenesis; females are more responsive than males. Of importance is our novel finding that 250 ng/L of 17α-ethinylestradiol (EE_2) depresses temperature-induced gonadal growth in males.

We also analyzed the effect of salinity and EE_2 on steroidogenesis. Salinity did not affect mummichog reproductive endocrine status, although EE_2 had a more prominent effect on female circulating 17β-estradiol (E_2) at 16 ppt than 32 ppt or 0 ppt salinity. As explained by Blewett (2011), EE_2 uptake varies with salinity as uptake is higher at 16 ppt, than at 0 ppt or 32 ppt, due to variations in gill thickness. At 16 ppt the diffusion distance is smaller than at 0 ppt or 32 ppt due to the presence of apical pores on the outer lamellae. At 0 ppt there is a predominance of thick cuboidal cells intermingled with pavement cells and dormant seawater chlorine cells, while at 32 ppt there is an increased number of seawater chloride cells.

EE_2 at 250 ng/L depressed E_2 production in females and 11-ketotestosterone (11-KT) production in males. Using steroidogenic precursors, we demonstrated that the effect of EE_2 is as far downstream as testosterone (T) to E_2 conversion in females and T to 11-KT in males, corroborating and furthering the work of Hogan *et al.* (2010). However, we were not able to demonstrate an effect of EE_2 on aromatase A gene expression. In summary, while some hypotheses were accepted, others were rejected (Table 3.1).

Table 3.1: Summary of the acceptance or rejection of the hypotheses postulated for this study. E_2 = 17β-Estradiol; 11-KT = 11-ketotestoterone; T = Testosterone; EE_2 = 17α-Ethinylestradiol; GSI = Gonadosomatic indices; LSI = Liver somatic indices

Null Hypothesis	Accepted (A) or Rejected (R)	Reasons for Rejection
EE_2 does not affect organ weight and somatic indices	R	↓ in ♂ GSI when held at 26°C
Salinity (or temperature) does not affect organ weight and somatic indices	(Salinity) R	↑ in ♂ organ weight and somatic indices
	(Temp.) R	↑ in ♀ and ♂ GSI and gonad weight
There is no interaction of EE_2 and salinity (or temperature) on organ weight and somatic indices	(Salinity) R	Interaction between salinity and EE_2 for ♀ LSI
	(Temp.) R	Interaction between temperature and EE_2 for ♂ GSI
EE_2 does not affect circulating steroid levels	R	↓ in ♀ plasma E_2 ↓ in ♂ plasma 11-KT
Salinity (or temperature) does not affect circulating steroid levels	(Salinity) A	
	(Temp) R	↑ in ♀ plasma E_2 ↓ in ♂ plasma 11-KT
There is no interaction of EE_2 and salinity (or temperature) on circulating steroid levels	A	
EE_2 does not affect biosynthesis of gonadal terminal steroids	R	↓ in ♀ E_2 production ↓ in ♂ 11-KT production
Salinity (or temperature) does not affect biosynthesis of gonadal terminal steroids	(Salinity) R	↓ in ♂ 11-KT production
	(Temp.) R	↓ in ♂ 11-KT production ↑ in ♀ E_2 production
There is no interaction of EE_2 and salinity (or temperature) on biosynthesis of gonadal terminal steroids	(Salinity) R	Interaction between salinity and EE_2 ♀ and ♂ T production
	(Temp.) R	Interaction between temperature and EE_2 ♀ and ♂ T production
EE_2 does not affect P450 aromatase A (Cyp19a) gene expression	A	

3.2. Future directions

It is presumed that the 50 ng/L exposure was in the range of environmental relevance as EE_2 concentration downstream of sewage treatment plants range from 0.2 to 42 ng/L (Desbrow *et al*., 1998; Ternes *et al.*, 1999). The 250 ng/L is considered to be a pharmacological concentration and was used as a tool to help determine potential mechanisms of action. The lack of effect of EE_2 on aromatase A mRNA expression suggests that the effect of EE_2 are non-genomic and/or possibly post transcriptional. In the future it would be interesting to do further studies at the molecular level and to compare genomic and non genomic mechanisms of EE_2 action. EE_2 interacts with the estrogen receptor (ER) causing endocrine disruption by mimicking the action of the endogenous E_2 at the receptor (Segner *et al.*, 2003). Orlando *et al*. (2006) found that the ER gene structure varies between species. Consequently, it would be interesting to add an inter-specific analysis to determine if EE_2 interaction with the ER differs among species.

EE_2 is a potent estrogenic chemical, which has gained attention from researchers and society, because of its environmental relevance and its disruptive action. Effects of EE_2 as a model estrogen can be compared to other estrogenic, anti-estrogenic, androgenic and anti-androgenic EDSs (MacLatchy *et al.,* 2003; Sharpe *et al*., 2004). For instance, Atlantic cod (*Gadus morhua*) previtellogenic oocytes exposed to 50 and 100 μM of nonylphenol had reduced steroidogenic acute regulatory protein and P450 side-chain cleavage expression and plasma E_2 and 11-KT after 14 d of exposure (Kortner & Arukwe, 2007). Flounder (*Platichthys flesus*. L) ovarian vitellogenic tissue exposed to 15 μM of phenanthrene, benzo[a]pyrene or chrysene for 24 h, had inhibited on 17,20-lyase,

17β-hydroxysteroid dehydrogenase and aromatase activity (Rocha-Monteiro *et al.*, 2000a). Additionally, flounder showed a significant decrease in circulating E_2, when exposed *in vivo* to 0.5, 2.5 and 12.5 nmol of phenanthrene/g of food for 12 weeks (Rocha-Monteiro *et al*., 2000b). Rutherford (2011) found that mummichog exposed to 100 and 1000 ng/L of the androgen 17α-methyl testosterone or 10 and 100 μg/L of dihydrotestosterone caused decreased circulating E_2 and 100 μg/L of dihydrotestosterone produced a depression of *in vitro* ovarian E_2 production. The effects of these estrogenic, antiestrogenic and androgenic compounds are comparable to EE_2's estrogenic effects, however, differences do exist, including among estrogens. Legler *et al.* (2002) compared EE_2's effect with those of E_2, estrone, nonylphenol and di(2-ethylhexyl)phthalate by exposing zebrafish (*Danio rerio*). They found that EE_2 had 100 times more estrogenic potency than E_2 and nonylphenol. Our work and these previous studies encourage focusing efforts on understanding the effects of endocrine disrupting substances (EDSs) on steroidogenesis and comparing different EDSs to elucidate the mechanisms of their toxicological effects.

3.3 Integrative approach

By analyzing the effects of EE_2 at different levels of biological organization we were able to demonstrate that EE_2 affects general reproductive endocrine status in mummichog. EE_2 effects on gonadal steroidogenesis may have consequent effects on circulating levels of terminal steroids in female and in male mummichog (Figure 3.1). Although population-level effects were not directly studied, links between reproductive

status and population impacts have been demonstrated elsewhere (Kidd *et al*., 2007). By evaluating the effect of environmental factors and EE_2 on mummichog reproductive physiology, we were able to give to our study a relevant environmental perspective missing from many laboratory studies.

Our work may be of interest in regard to sewage treatment plant (STP) management, as it indicates that EE_2 present in STP effluents is potentially disruptive for reproductive endocrine status in fish, in particular mummichog. Our results suggest that, in addition to developing improved STP treatment for estrogenic contaminants, temporal and spatial release of sewage should be studied in association with environmental parameters and fish reproductive cycles so as to reduce the impact on estuarine fish species.

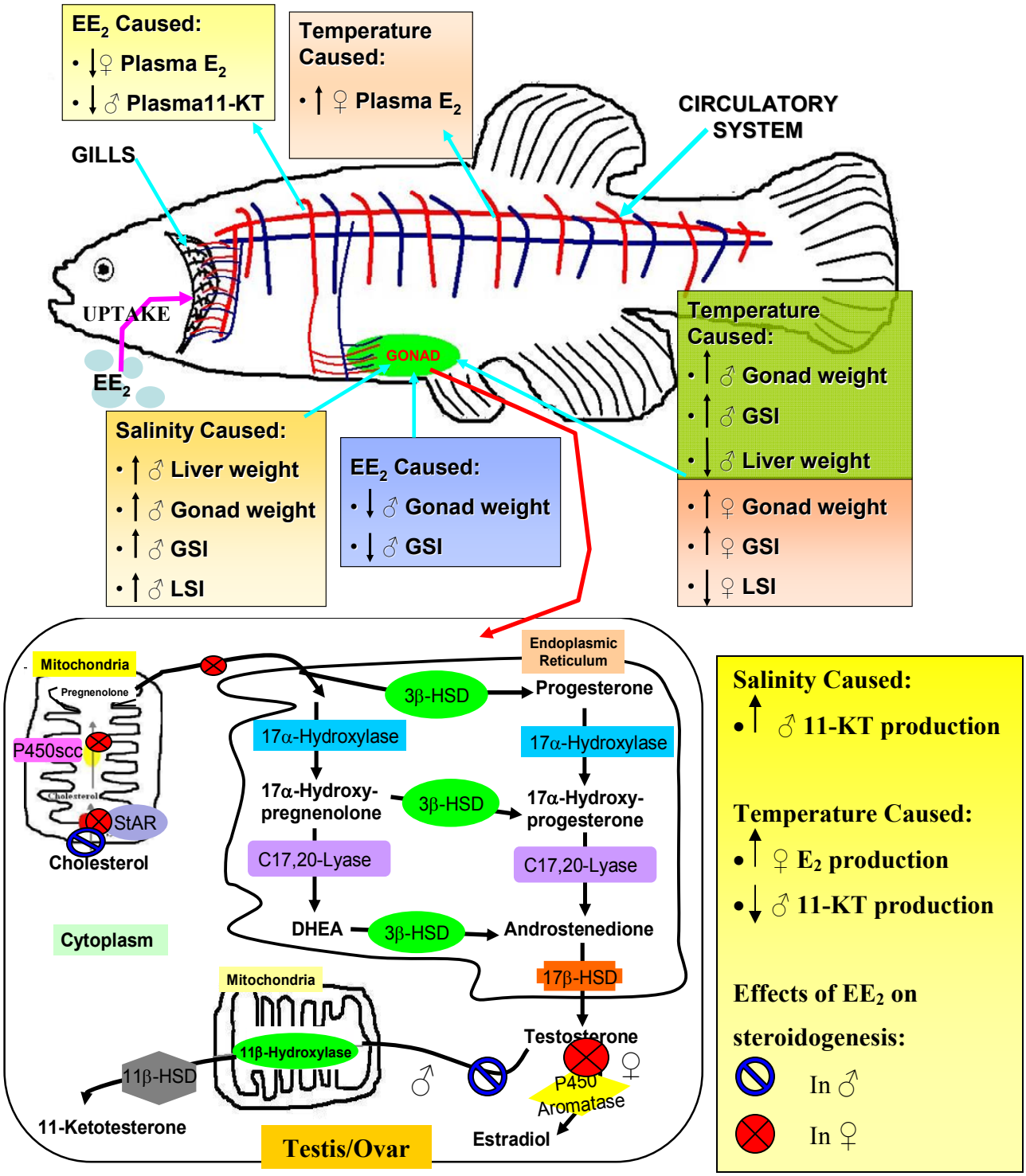

Figure 3.1: Summary scheme of the effects of salinity, temperature and 17α-ethinylestradiol (EE_2) on mummichog (*Fundulus heteroclitus*) reproductive endocrine status. Steroidogenic pathway modified from McMaster *et al.*, 1995 & Leusch, 2001. EE_2 uptake is via the gills (Blewett, 2011). E_2 = 17β-Estradiol; 11-KT = 11-Ketotestosterone; LSI = Liver somatic index; GSI= Gonadosomatic index.

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